

A PHARMACEUTICAL COMPOSITION COMPRISING AN IMMUNOGLOBULIN
FC REGION AS A CARRIER

Technical Field

5 The present invention relates to a novel use of an immunoglobulin Fc fragment. More particularly, the present invention relates to a pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier, and a method of improving the in vivo duration of action of a drug linked to an immunoglobulin Fc fragment.

10 Background Art

In the past, a large number of pharmacologists and chemists made efforts to chemically alter and/or modify the in vivo activity of naturally existing, physiologically active molecules. These efforts mainly focused on 15 increasing or prolonging certain in vivo activity, reducing toxicity, eliminating or reducing side effects, or modifying specific physiological activities of the physiologically active substances. When a physiologically active substance is chemically modified, it loses some or 20 most of its physiological activities in many cases. However, in some cases, the modification could result in an increase or change in physiological activity. In this

regard, many studies have been focused on chemical modification capable of achieving desired physiological activity, and most of such studies have involved covalently bonding a physiologically active substance (drug) to a 5 physiologically acceptable carrier.

For example, International Pat. Publication No. WO 01/93911 employs a polymer having a plurality of acid moieties as a drug carrier. International Pat. Publication No. WO 03/00778, discloses an anionic group-containing 10 amphiphilic block copolymers that, when used as a drug carrier for a cationic drug, improve the stability of the drug. European Pat. No. 0 681 481 describes a method of improving the properties of basic drugs by using cyclodextrin and acids as carriers. On the other hand, hydrophobic drugs 15 have low stability in vivo mainly due to their low aqueous solubility. To improve the low aqueous solubility of hydrophobic drugs, International Pat. Publication No. WO 04/064731 employs a lipid as a carrier. However, to date, there is no report for the use of an immunoglobulin Fc 20 fragment as a drug carrier.

Typically, since polypeptides are relatively easily denatured due to their low stability, degraded by proteolytic enzymes in the blood and easily eliminated through the kidney or liver, protein medicaments, including polypeptides as 25 pharmaceutically effective components, need to be frequently administered to patients to maintain desired blood level

concentrations and titers. However, this frequent administration of protein medicaments, especially through injection causes pain for patients. To solve these problems, many efforts have been made to improve the serum stability of 5 protein drugs and maintain the drugs in the blood at high levels for a prolonged period of time, and thus maximize the pharmaceutical efficacy of the drugs. Pharmaceutical compositions with sustained activity, therefore, need to increase the stability of protein drugs and maintain the 10 titers at sufficiently high levels without causing immune responses in patients.

To stabilize proteins and prevent enzymatic degradation and clearance by the kidneys, a polymer having high solubility, such as polyethylene glycol (hereinafter, 15 referred to simply as "PEG"), was conventionally used to chemically modify the surface of a protein drug. By binding to specific or various regions of a target protein, PEG stabilizes the protein and prevents hydrolysis, without causing serious side effects (Sada et al., *J. Fermentation Bioengineering* 71: 137-139, 1991). However, despite its 20 capability to enhance protein stability, this PEG coupling has problems such as greatly reducing the number titers of physiologically "active" proteins. Further the yield decreases with the increasing molecular weight of PEG due to 25 the reduced reactivity with the proteins.

Recently, polymer-protein drug conjugates have been

suggested. For example, as described in U.S. Pat. No. 5,738,846, a conjugate can be prepared by linking an identical protein drug to both ends of PEG to improve the activity of the protein drug. Also, as described in 5 International Pat. Publication No. WO 92/16221, two different protein drugs can be linked to both ends of PEG to provide a conjugate having two different activities. The above methods, however, were not very successful in sustaining the activity of protein drugs.

10 On the other hand, Kinstler et al. reported that a fusion protein prepared by coupling granulocyte-colony stimulating factor (G-CSF) to human albumin showed improved stability (Kinstler et al., *Pharmaceutical Research* 12(12): 1883-1888, 1995). In this publication, however, since the 15 modified drug, having a G-CSF-PEG-albumin structure, only showed an approximately four-fold increase in residence time in the body and a slight increase in serum half-life compared to the single administration of the native G-CSF, it has not been industrialized as an effective long-acting formulation 20 for protein drugs.

An alternative method for improving the *in vivo* stability of physiologically active proteins is by linking a gene of physiologically active protein to a gene encoding a protein having high serum stability by genetic 25 recombination technology and culturing the cells transfected with the recombinant gene to produce a fusion

protein. For example, a fusion protein can be prepared by conjugating albumin, a protein known to be the most effective in enhancing protein stability, or its fragment to a physiologically active protein of interest by genetic recombination (International Pat. Publication Nos. WO 93/15199 and WO 93/15200, European Pat. Publication No. 413,622). A fusion protein of interferon-alpha and albumin, developed by the Human Genome Science Company and marketed under the trade name of 'AlbuferonTM', increased the half-life from 5 hours to 93 hours in monkeys, but it was known to be problematic because it decreased the in vivo activity to less than 5% of unmodified interferon-alpha (Osborn et al., *J. Phar. Exp. Ther.* 303(2): 540-548, 2002).

Recombinant DNA technologies were applied to fuse a protein drug to an immunoglobulin Fc fragment. For example, interferon (Korean Pat. Laid-open Publication No. 2003-9464), and interleukin-4 receptor, interleukin-7 receptor or erythropoietin (EPO) receptor (Korean Pat. Registration No. 249572) were previously expressed in mammals in a form fused to an immunoglobulin Fc fragment. International Pat. Publication No. WO 01/03737 describes a fusion protein comprising a cytokine or growth factor linked to an immunoglobulin Fc fragment through peptide linkage. In addition, U.S. Pat No. 5,116,964 discloses proteins fused to the amino- or carboxyl-terminal end of an immunoglobulin Fc fragment by genetic recombination. U.S. Pat. No. 5,349,053

discloses a fusion protein comprising IL-2 fused to an immunoglobulin Fc fragment through peptide linkage. Other examples of Fc fusion proteins prepared by genetic recombination include a fusion protein of interferon-beta 5 or its derivative and an immunoglobulin Fc fragment (International Pat. Publication NO. WO 00/23472), and a fusion protein of IL-5 receptor and an immunoglobulin Fc fragment (U.S. Pat. NO. 5,712,121), a fusion protein of interferon alpha and the Fc fragment of immunoglobulin G4 10 (U.S. Pat. No. 5,723,125), and a fusion protein of CD4 protein and the Fc fragment of immunoglobulin G2 (U.S. Pat. No. 6,451,313).

Techniques involving the modification of amino acid residues of an immunoglobulin Fc fragment are also known. 15 For example, U.S. Pat. NO. 5,605,690 discloses a TNFR-IgG1 Fc fusion protein, which is prepared by genetic recombination using an IgG1 Fc fragment having amino acid alterations in the complement binding region or receptor binding region. Also, other methods of preparing a fusion protein using a 20 modified immunoglobulin Fc fragment by genetic recombination are disclosed in U.S. Pat. Nos. 6,277,375, 6,410,008 and 6,444,792.

Such Fc fusion proteins produced by genetic recombination have the following disadvantages: protein 25 fusion occurs only in a specific region of an immunoglobulin Fc fragment, which is at an amino- or

carboxyl-terminal end; only homodimeric forms and not monomeric forms are produced; and a fusion could take place only between the glycosylated proteins or between the aglycosylated proteins, and it is impossible to make a 5 fusion protein composed of a glycosylated protein and an aglycosylated protein. Further, a new amino acid sequence created by the fusion may trigger immune responses, and a linker region may become susceptible to proteolytic degradation.

10 To solve these problems, the inventors of the present application conducted a research, and came to a knowledge that, when a drug is administered in the form of being linked to an IgG Fc fragment, the drug has improved in vivo stability while exhibiting a minimal reduction in the in vivo 15 activity.

Disclosure of the Invention

It is therefore an object of the present invention to provide a pharmaceutical composition comprising an immunoglobulin Fc fragment as a carrier.

20 It is another object of the present invention to provide a method for improving the in vivo duration of action of a drug by including an immunoglobulin Fc fragment as a carrier.

Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 shows the results of chromatography of an immunoglobulin Fc fragment obtained by cleavage of an immunoglobulin with papain;

FIG. 2 shows the results of SDS-PAGE of a purified immunoglobulin Fc fragment (M: molecular size marker, lane 1: IgG, lane 2: Fc);

FIG. 3 shows the results of SDS-PAGE of IFN α -PEG-Fc (A), 17 Ser-G-CSF-PEG-Fc (B) and EPO-PEG-Fc (C) conjugates, which are generated by a coupling reaction (M: molecular size marker, lane 1: Fc, lane 2: physiologically active protein, lane 3: physiologically active protein-PEG-Fc conjugate);

FIG. 4 shows the results of size exclusion chromatography of an IFN α -PEG-Fc conjugate that is purified after a coupling reaction;

FIG. 5 shows the results of MALDI-TOF mass spectrometry of an EPO-PEG-Fc conjugate;

FIGS. 6a and 6b show the results of MALDI-TOF mass spectrometry and SDS-PAGE analysis, respectively, of a native immunoglobulin Fc and a deglycosylated

immunoglobulin Fc (DG Fc);

FIG. 7 shows the results of MALDI-TOF mass spectrometry of an IFN α -PEG-Fc conjugate and an IFN α -PEG-DG Fc conjugate;

5 FIGS. 8a to 8c show the results of reverse phase HPLC of IFN α -PEG-Fc, IFN α -PEG-DG Fc and IFN α -PEG-recombinant AG Fc derivative conjugates;

10 FIG. 9 is a graph showing the results of pharmacokinetic analysis of a native IFN α , an IFN α -40K PEG complex, an IFN α -PEG-albumin conjugate and an IFN α -PEG-Fc conjugate;

15 FIG. 10 is a graph showing the results of pharmacokinetic analysis of a native EPO, a highly glycosylated EPO, an EPO-PEG-Fc conjugate and an EPO-PEG-AG Fc conjugate;

FIG. 11 is a graph showing the results of pharmacokinetic analysis of IFN α -PEG-Fc, IFN α -PEG-DG Fc and IFN α -PEG-recombinant AG Fc conjugates;

20 FIG. 12 is a graph showing the pharmacokinetics of a Fab', a Fab'-S-40K PEG complex, a Fab'-N-PEG-N-Fc conjugate and a Fab'-S-PEG-N-Fc conjugate;

FIG. 13 is a graph showing the in vivo activities of Fab', a Fab'-S-40K PEG complex, a Fab'-N-PEG-N-Fc conjugate and a Fab'-S-PEG-N-Fc conjugate;

25 FIG. 14 is a graph showing the results of comparison of human IgG subclasses for binding affinity to the C1q

complement; and

FIG. 15 is a graph showing the results of comparison of a glycosylated Fc, an enzymatically deglycosylated DG Fc and an interferon-PEG-carrier conjugate where the carrier 5 is AG Fc produced by *E. coli* for binding affinity to the C1q complement.

Best Mode for Carrying Out the Invention

In one aspect, the present invention relates to a pharmaceutical composition comprising an immunoglobulin Fc 10 fragment as a carrier.

The term "carrier", as used herein, refers to a substance linked with a drug, which typically increases, decreases or eliminates the physiological activity of the drug by binding to the drug. However, with respect to the 15 objects of the present invention, a carrier is employed in the present invention for minimizing a decrease in the physiological activity of a drug of interest, linked to the carrier, while enhancing the in vivo stability of the drug.

To accomplish the objects of the present invention, 20 the present invention is characterized by employing an immunoglobulin Fc fragment as a carrier.

The immunoglobulin Fc fragment is safe for use as a drug carrier because it is a biodegradable polypeptide that is metabolized in the body. Also, the immunoglobulin Fc

fragment has a relatively low molecular weight compared to the whole immunoglobulin molecule, thus being advantageous in the preparation, purification and yield of conjugates due to. Further, since the immunoglobulin Fc fragment does 5 not contain the Fab fragment, whose amino acid sequence differs among antibody subclasses and which thus is highly non-homogenous, it may greatly increase the homogeneity of substances and be less antigenic.

The term "immunoglobulin Fc fragment", as used 10 herein, refers to a protein that contains the heavy-chain constant region 2 (C_H2) and the heavy-chain constant region 3 (C_H3) of an immunoglobulin, and not the variable regions of the heavy and light chains, the heavy-chain constant region 1 (C_H1) and the light-chain constant region 1 (C_L1) 15 of the immunoglobulin. It may further include the hinge region at the heavy-chain constant region. Also, the immunoglobulin Fc fragment of the present invention may contain a portion or the all the heavy-chain constant region 1 (C_H1) and/or the light-chain constant region 1 (C_L1), except for the variable regions of the heavy and 20 light chains. Also, as long as it has a physiological function substantially similar to or better than the native protein the IgG Fc fragment may be a fragment having a deletion in a relatively long portion of the amino acid 25 sequence of C_H2 and/or C_H3 . That is, the immunoglobulin Fc fragment of the present invention may comprise 1) a C_H1

domain, a C_H2 domain, a C_H3 domain and a C_H4 domain, 2) a C_H1 domain and a C_H2 domain, 3) a C_H1 domain and a C_H3 domain, 4) a C_H2 domain and a C_H3 domain, 5) a combination of one or more domains and an immunoglobulin hinge region (or a portion of the hinge region), and 6) a dimer of each domain of the heavy-chain constant regions and the light-chain constant region.

The Fc fragment of the present invention includes a native amino acid sequence and sequence derivatives (mutants) thereof. An amino acid sequence derivative is a sequence that is different from the native amino acid sequence due to a deletion, an insertion, a non-conservative or conservative substitution or combinations thereof of one or more amino acid residues. For example, in an IgG Fc, amino acid residues known to be important in binding, at positions 214 to 238, 297 to 299, 318 to 322, or 327 to 331, may be used as a suitable target for modification. Also, other various derivatives are possible, including one in which a region capable of forming a disulfide bond is deleted, or certain amino acid residues are eliminated at the N-terminal end of a native Fc form or a methionine residue is added thereto. Further, to remove effector functions, a deletion may occur in a complement-binding site, such as a C1q-binding site and an ADCC site. Techniques of preparing such sequence derivatives of the immunoglobulin Fc fragment are disclosed in International

Pat. Publication Nos. WO 97/34631 and WO 96/32478.

Amino acid exchanges in proteins and peptides, which do not generally alter the activity of the proteins, or peptides are known in the art (H. Neurath, R. L. Hill, *The Proteins*, Academic Press, New York, 1979). The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Thy/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu and Asp/Gly, in both directions.

In addition, the Fc fragment, if desired, may be modified by phosphorylation, sulfation, acylation, glycosylation, methylation, farnesylation, acetylation, amidation, and the like.

The aforementioned Fc derivatives are derivatives that have a biological activity identical to the Fc fragment of the present invention or improved structural stability, for example, against heat, pH, or the like.

In addition, these Fc fragments may be obtained from native forms isolated from humans and other animals including cows, goats, swine, mice, rabbits, hamsters, rats and guinea pigs, or may be recombinants or derivatives thereof, obtained from transformed animal cells or microorganisms. Herein, they may be obtained from a native immunoglobulin by isolating whole immunoglobulins from human or animal organisms and treating them with a proteolytic enzyme. Papain digests the native

immunoglobulin into Fab and Fc fragments, and pepsin treatment results in the production of pF'c and F (ab') 2 fragments. These fragments may be subjected, for example, to size exclusion chromatography to isolate Fc or pF'c.

5 Preferably, a human-derived Fc fragment is a recombinant immunoglobulin Fc fragment that is obtained from a microorganism.

In addition, the immunoglobulin Fc fragment of the present invention may be in the form of having native sugar 10 chains, increased sugar chains compared to a native form or decreased sugar chains compared to the native form, or may be in a deglycosylated form. The increase, decrease or removal of the immunoglobulin Fc sugar chains may be achieved by methods common in the art, such as a chemical 15 method, an enzymatic method and a genetic engineering method using a microorganism. The removal of sugar chains from an Fc fragment results in a sharp decrease in binding affinity to the C1q part of the first complement component C1 and a decrease or loss in antibody-dependent cell- 20 mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), thereby not inducing unnecessary immune responses in vivo. In this regard, an immunoglobulin Fc fragment in a deglycosylated or aglycosylated form may be more suitable to the object of the present invention as a 25 drug carrier.

As used herein, the term "deglycosylation" refers to

that sugar moieties are enzymatically removed from an Fc fragment, and the term "aglycosylation" means that an Fc fragment is produced in an unglycosylated form by a prokaryote, preferably *E. coli*.

5 On the other hand, the immunoglobulin Fc fragment may be derived from humans or other animals including cows, goats, swine, mice, rabbits, hamsters, rats and guinea pigs, and preferably humans. In addition, the immunoglobulin Fc fragment may be an Fc fragment that is 10 derived from IgG, IgA, IgD, IgE and IgM, or that is made by combinations thereof or hybrids thereof. Preferably, it is derived from IgG or IgM, which is among the most abundant proteins in human blood, and most preferably from IgG, which is known to enhance the half-lives of ligand-binding 15 proteins.

On the other hand, the term "combination", as used herein, means that polypeptides encoding single-chain immunoglobulin Fc fragments of the same origin are linked to a single-chain polypeptide of a different origin to form 20 a dimer or multimer. That is, a dimer or multimer may be formed from two or more fragments selected from the group consisting of IgG1 Fc, IgG2 Fc, IgG3 Fc and IgG4 Fc fragments.

The term "hybrid", as used herein, means that 25 sequences encoding two or more immunoglobulin Fc fragments of different origin are present in a single-chain

immunoglobulin Fc fragment. In the present invention, various types of hybrids are possible. That is, domain hybrids may be composed of one to four domains selected from the group consisting of CH1, CH2, CH3 and CH4 of IgG1 Fc, IgG2 Fc, IgG3 Fc and IgG4 Fc, and may include the hinge region.

On the other hand, IgG is divided into IgG1, IgG2, IgG3 and IgG4 subclasses, and the present invention includes combinations and hybrids thereof. Preferred are IgG2 and IgG4 subclasses, and most preferred is the Fc fragment of IgG4 rarely having effector functions such as CDC (complement dependent cytotoxicity) (see, FIGS. 14 and 15).

That is, as the drug carrier of the present invention, the most preferable immunoglobulin Fc fragment is a human IgG4-derived non-glycosylated Fc fragment. The human-derived Fc fragment is more preferable than a non-human derived Fc fragment, which may act as an antigen in the human body and cause undesirable immune responses such as the production of a new antibody against the antigen.

The immunoglobulin Fc fragment of the present invention, prepared as described above, acts as a drug carrier and forms a conjugate with a drug.

The term "drug conjugate" or "conjugate", as used herein, means that one or more drugs are linked with one or more immunoglobulin Fc fragments.

The term "drug", as used herein, refers to a substance displaying therapeutic activity when administered to humans or animals, and examples of the drug include, but are not limited to, polypeptides, compounds, extracts and 5 nucleic acids. Preferred is a polypeptide drug.

The terms "physiologically active polypeptide", "physiologically active protein", "active polypeptide" "polypeptide drug" and "protein drug", as used herein, are interchangeable in their meanings, and are featured in that 10 they are in a physiologically active form exhibiting various in vivo physiological functions.

The polypeptide drug has a disadvantage of being unable to sustain physiological action for a long period of time due to its property of being easily denatured or 15 degraded by proteolytic enzymes present in the body. However, when the polypeptide drug is conjugated to the immunoglobulin Fc fragment of the present invention to form a conjugate, the drug has increased structural stability and degradation half-life. Also, the polypeptide conjugated 20 to the Fc fragment has a much smaller decrease in physiological activity than other known polypeptide drug formulations. Therefore, compared to the in vivo bioavailability of conventional polypeptide drugs, the conjugate of the polypeptide and the immunoglobulin Fc 25 fragment according to the present invention is characterized by having markedly improved in vivo

bioavailability. This is also clearly described through embodiments of the present invention. That is, when linked to the immunoglobulin Fc fragment of the present invention, IFN α , G-CSF, hGH and other protein drugs displayed an about 5 two- to six-fold increase in vivo bioavailability compared to their conventional forms conjugated to PEG alone or both PEG and albumin (Tables 8, 9 and 10).

On the other hand, the linkage of a protein and the immunoglobulin Fc fragment of the present invention is 10 featured in that it is not a fusion by a conventional recombination method. A fusion form of the immunoglobulin Fc fragment and an active polypeptide used as a drug by a recombination method is obtained in such a way that the polypeptide is linked to the N-terminus or C-terminus of 15 the Fc fragment, and is thus expressed and folded as a single polypeptide from a nucleotide sequence encoding the fusion form.

This brings about a sharp decrease in the activity of 20 the resulting fusion protein because the activity of a protein as a physiologically functional substance is determined by the conformation of the protein. Thus, when a polypeptide drug is fused with Fc by a recombination method, there is no effect with regard to in vivo 25 bioavailability even when the fusion protein has increased structural stability. Also, since such a fusion protein is often misfolded and thus expressed as inclusion bodies, the

fusion method is uneconomical in protein production and isolation yield. Further, when the active form of a polypeptide is in a glycosylated form, the polypeptide should be expressed in eukaryotic cells. In this case, Fc 5 is also glycosylated, and this glycosylation may cause unsuitable immune responses *in vivo*.

That is, only the present invention makes it possible to produce a conjugate of a glycosylated active polypeptide and an aglycosylated immunoglobulin Fc fragment, and 10 overcomes all of the above problems, including improving protein production yield, because the two components of the complex are individually prepared and isolated by the best systems.

Non-limiting examples of protein drugs capable of 15 being conjugated to the immunoglobulin Fc fragment of the present invention include human growth hormone, growth hormone releasing hormone, growth hormone releasing peptide, interferons and interferon receptors (e.g., interferon- α , - β and - γ , water-soluble type I interferon 20 receptor, etc.), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), glucagon-like peptides (e.g., GLP-1, etc.), G- 25 protein-coupled receptor, interleukins (e.g., interleukin-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, -17, -18, -19, -20, -21, -22, -23, -24, -25, -26, -27, -28, -29, -30, etc.) and interleukin receptors (e.g.,

IL-1 receptor, IL-4 receptor, etc.), enzymes (e.g., glucocerebrosidase, iduronate-2-sulfatase, alpha-galactosidase-A, agalsidase alpha and beta, alpha-L-iduronidase, butyrylcholinesterase, chitinase, glutamate decarboxylase, imiglucerase, lipase, uricase, platelet-activating factor acetylhydrolase, neutral endopeptidase, myeloperoxidase, etc.), interleukin and cytokine binding proteins (e.g., IL-18bp, TNF-binding protein, etc.), macrophage activating factor, macrophage peptide, B cell factor, T cell factor, protein A, allergy inhibitor, cell necrosis glycoproteins, immunotoxin, lymphotoxin, tumor necrosis factor, tumor suppressors, metastasis growth factor, alpha-1 antitrypsin, albumin, alpha-lactalbumin, apolipoprotein-E, erythropoietin, highly glycosylated erythropoietin, angiopoietins, hemoglobin, thrombin, thrombin receptor activating peptide, thrombomodulin, factor VII, factor VIIa, factor VIII, factor IX, factor XIII, plasminogen activating factor, fibrin-binding peptide, urokinase, streptokinase, hirudin, protein C, C-reactive protein, renin inhibitor, collagenase inhibitor, superoxide dismutase, leptin, platelet-derived growth factor, epithelial growth factor, epidermal growth factor, angiostatin, angiotensin, bone growth factor, bone stimulating protein, calcitonin, insulin, atriopeptin, cartilage inducing factor, elcatonin, connective tissue activating factor, tissue factor pathway inhibitor,

follicle stimulating hormone, luteinizing hormone, luteinizing hormone releasing hormone, nerve growth factors (e.g., nerve growth factor, ciliary neurotrophic factor, axogenesis factor-1, brain-natriuretic peptide, glial 5 derived neurotrophic factor, netrin, neurophil inhibitor factor, neurotrophic factor, neuturin, etc.), parathyroid hormone, relaxin, secretin, somatomedin, insulin-like growth factor, adrenocortical hormone, glucagon, cholecystokinin, pancreatic polypeptide, gastrin releasing peptide, corticotropin releasing factor, thyroid 10 stimulating hormone, autotaxin, lactoferrin, myostatin, receptors (e.g., TNFR(P75), TNFR(P55), IL-1 receptor, VEGF receptor, B cell activating factor receptor, etc.), receptor antagonists (e.g., IL1-Ra etc.), cell surface 15 antigens (e.g., CD 2, 3, 4, 5, 7, 11a, 11b, 18, 19, 20, 23, 25, 33, 38, 40, 45, 69, etc.), virus vaccine antigens, monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., scFv, Fab, Fab', F(ab')2 and Fd), and virus 20 derived vaccine antigens. An antibody fragment may be Fab, Fab', F (ab') 2, Fd or scFv, which is capable of binding to a specific antigen, and preferably Fab'. The Fab fragments contain the variable domain (V_L) and const domain (C_L) of the light chain and the variable domain (V_H) and the first 25 constant domain (C_H1) of the heavy chain. The Fab' fragments differ from the Fab fragments in terms of adding several amino acid residues including one or more cysteine residues

from the hinge region to the carboxyl terminus of the C_{H1} domain. The Fd fragments comprise only the V_H and C_{H1} domain, and the F (ab') 2 fragments are produced as a pair of Fab' fragments by either disulfide bonding or a chemical reaction. The scFv (single-chain Fv) fragments comprise the V_L and V_H domains that are linked to each other by a peptide linker and thus are present in a single polypeptide chain.

In particular, preferred as physiologically active polypeptides are those requiring frequent dosing upon administration to the body for therapy or prevention of diseases, which include human growth hormone, interferons (interferon- α , - β , - γ , etc.), granulocyte colony stimulating factor, erythropoietin (EPO) and antibody fragments. In addition, certain derivatives are included in the scope of the physiologically active polypeptides of the present invention as long as they have function, structure, activity or stability substantially identical to or improved compared over native forms of the physiologically active polypeptides. In the present invention, the most preferable polypeptide drug is interferon-alpha.

In addition to the polypeptide drugs, other drugs are also available in the present invention. Non-limiting examples of these drugs include antibiotics selected from among derivatives and mixtures of tetracycline, 25 minocycline, doxycycline, ofloxacin, levofloxacin, ciprofloxacin, clarithromycin, erythromycin, cefaclor,

cefotaxime, imipenem, penicillin, gentamycin, streptomycin, vancomycin, and the like; anticancer agents selected from among derivatives and mixtures of methotrexate, carboplatin, taxol, cisplatin, 5-fluorouracil, doxorubicin, 5 etoposide, paclitaxel, camtotecin, cytosine arabinoside, and the like; anti-inflammatory agents selected from among derivatives and mixtures of indomethacin, ibuprofen, ketoprofen, piroxicam, probiprofen, diclofenac, and the like; antiviral agents selected from among derivatives and 10 mixtures of acyclovir and robavine; and antibacterial agents selected from among derivatives and mixtures of ketoconazole, itraconazole, fluconazole, amphotericin B and griseofulvin.

On the other hand, the immunoglobulin Fc fragment of 15 the present invention is able to form a conjugate linked to a drug through a linker.

This linker includes peptide and non-peptide linkers. Preferred is a non-peptide linker, and more preferred is a non-peptide polymer.

20 The term "peptide linker", as used herein, means amino acids, and preferably 1 to 20 amino acids, which are linearly linked to each other by peptide bonding, and may be in a glycosylated form. With respect to the objects of the present invention, preferred is an aglycosylated form. 25 This peptide linker is preferably a peptide having a repeating unit of Gly and Ser, which is immunologically

inactive for T cells.

The term "non-peptide polymer", as used herein, refers to a biocompatible polymer including two or more repeating units linked to each other by a covalent bond 5 excluding the peptide bond. Examples of the non-peptide polymer include poly (ethylene glycol), poly (propylene glycol), copolymers of ethylene glycol and propylene glycol, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, dextran, polyvinyl ether, biodegradable 10 polymers such as PLA (poly (lactic acid) and PLGA (poly (lactic-glycolic acid), lipid polymers, chitins, and hyaluronic acid. The most preferred is poly (ethylene glycol) (PEG).

The conjugate of the present invention, 15 immunoglobulin Fc fragment-drug or immunoglobulin Fc fragment-linker-drug, is made at various molar ratios. That is, the number of the immunoglobulin Fc fragment and/or linker linked to a single polypeptide drug is not limited. However, preferably, in the drug conjugate of the present 20 invention, the drug and the immunoglobulin Fc fragment are conjugated to each other at a molar ratio of 1:1 to 10:1, and preferably 1:1 to 2:1.

In addition, the linkage of the immunoglobulin Fc fragment of the present invention, a certain linker and a 25 certain drug include all covalent bonds except for a peptide bond formed when the Fc fragment and a polypeptide

drug are expressed as a fusion protein by genetic recombination, and all types of non-covalent bonds such as hydrogen bonds, ionic interactions, van der Waals forces and hydrophobic interactions. However, with respect to the 5 physiological activity of the drug, the linkage is preferably made by covalent bonds.

In addition, the immunoglobulin Fc fragment of the present invention, a certain linker and a certain drug may be linked to each other at a certain site of the drug. On 10 the other hand, the immunoglobulin Fc fragment of the present invention and a polypeptide drug may be linked to each other at an N-terminus or C-terminus, and preferably at a free group, and a covalent bond between the immunoglobulin Fc fragment and the drug is easily formed 15 especially at an amino terminal end, an amino group of a lysine residue, an amino group of a histidine residue, or a free cysteine residue.

On the other hand, the linkage of the immunoglobulin Fc fragment of the present invention, a certain linker and a certain drug may be made in a certain direction. That is, 20 the linker may be linked to the N-terminus, the C-terminus or a free group of the immunoglobulin Fc fragment, and may also be linked to the N-terminus, the C-terminus or a free group of the protein drug. When the linker is a peptide 25 linker, the linkage may take place at a certain linking site.

Also, the conjugate of the present invention may be prepared using any of a number of coupling agents known in the art. Non-limiting examples of the coupling agents include 1,1-bis (diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters such as esters with 4-azidosalicylic acid, imidoesters including disuccinimidyl esters such as 3,3'-dithiobis (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane.

On the other hand, the pharmaceutical composition of the present invention, comprising an immunoglobulin Fc fragment as a carrier, may be administered via a variety of routes.

The term "administration", as used herein, means introduction of a predetermined amount of a substance into a patient by a certain suitable method. The conjugate of the present invention may be administered via any of the common routes, as long as it is able to reach a desired tissue. A variety of modes of administration are contemplated, including intraperitoneally, intravenously, intramuscularly, subcutaneously, intradermally, orally, topically, intranasally, intrapulmonarily and intrarectally, but the present invention is not limited to these exemplified modes of administration. However, since peptides are digested upon oral administration, active ingredients of a composition for oral administration should

be coated or formulated for protection against degradation in the stomach. Preferably, the present composition may be administered in an injectable form. In addition, the pharmaceutical composition of the present invention may be administered using a certain apparatus capable of transporting the active ingredients into a target cell.

5 The pharmaceutical composition comprising the conjugate according to the present invention may include a pharmaceutically acceptable carrier. For oral administration, the pharmaceutically acceptable carrier may include binders, lubricants, disintegrators, excipients, solubilizers, dispersing agents, stabilizers, suspending agents, coloring agents and perfumes. For injectable preparations, the pharmaceutically acceptable carrier may include buffering agents, preserving agents, analgesics, 10 solubilizers, isotonic agents and stabilizers. For preparations for topical administration, the pharmaceutically acceptable carrier may include bases, excipients, lubricants and preserving agents. The 15 pharmaceutical composition of the present invention may be formulated into a variety of dosage forms in combination with the aforementioned pharmaceutically acceptable carriers. For example, for oral administration, the pharmaceutical composition may be formulated into tablets, 20 troches, capsules, elixirs, suspensions, syrups or wafers. For injectable preparations, the pharmaceutical composition 25

may be formulated into a unit dosage form, such as a multidose container or an ampule as a single-dose dosage form. The pharmaceutical composition may be also formulated into solutions, suspensions, tablets, capsules and long-
5 acting preparations.

On the other hand, examples of carriers, excipients and diluents suitable for the pharmaceutical formulations include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia rubber, alginate,
10 gelatin, calcium phosphate, calcium silicate, cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate and mineral oils. In addition, the pharmaceutical formulations may
15 further include fillers, anti-coagulating agents, lubricants, humectants, perfumes, emulsifiers and antiseptics.

A substantial dosage of a drug in combination with the immunoglobulin Fc fragment of the present invention as a carrier may be determined by several related factors including the types of diseases to be treated, administration routes, the patient's age, gender, weight and severity of the illness, as well as by the types of the drug as an active component. Since the pharmaceutical composition of the present invention has a very long duration of action in vivo,
20 it has an advantage of greatly reducing administration frequency of pharmaceutical drugs.

In another aspect, another object of the present invention is to provide a method of improving the in vivo duration of a drug by comprising an immunoglobulin Fc fragment.

5 In one embodiment of the present invention, a physiologically active polypeptide-PEG-immunoglobulin Fc fragment conjugate exerts much higher stability than a polypeptide-PEG complex or a polypeptide-PEG-albumin conjugate. Pharmacokinetic analysis revealed that IFN α has 10 a serum half-life increased by about 20 times when linked to 40-kDa PEG (IFN α -40K PEG complex) and by about 10 times in an IFN α -PEG-albumin conjugate, compared to the native IFN α . In contrast, an IFN α -PEG-Fc conjugate according to 15 the present invention showed a half-life remarkably increased by about 50 times (see, Table 3). In addition, the same result was observed in other target proteins, human growth hormone (hGH), granulocyte colony-stimulating factor (G-CSF) and its derivative (17 S-G-CSF), and erythropoietin (EPO). Protein conjugates according to the 20 present invention, each of which comprises a target protein linked to PEG-Fc, displayed increases about 10-fold in mean residence time (MRT) and serum half-life compared to the native forms of the proteins and the forms conjugated to PEG or PEG-albumin (see, Tables 4 to 7).

25 In addition, when a PEG-Fc complex is linked to an -SH group near the C-terminus of a Fab' or the N-terminus of

the Fab', the resulting Fab'-PEG-Fc conjugate displayed a 2 to 3-fold longer serum half-life than a 40K PEG-Fab' complex (see, FIG. 12).

Further, when protein conjugates are prepared using 5 deglycosylated immunoglobulin Fc (DG Fc), where sugar moieties are removed, and recombinant aglycosylated immunoglobulin Fc (AG Fc) derivatives, their plasma half-lives and in vitro activity were maintained similar to the protein conjugates prepared using the native Fc (see, Table 10 Table 3 and FIGS. 8 and 11).

Therefore, since the protein conjugates of the present invention have extended serum half-lives and mean residence time (MRT) when applied to a variety of physiologically active polypeptides including human growth 15 hormone, interferon, erythropoietin, colony stimulating factor or its derivatives, and antibody derivatives, they are useful for developing long-acting formulations of diverse physiologically active polypeptides.

A better understanding of the present invention may 20 be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limits of the present invention.

EXAMPLE 1: Preparation I of IFN α -PEG-immunoglobulin Fc fragment conjugate

<Step 1> Preparation of immunoglobulin Fc fragment using immunoglobulin

An immunoglobulin Fc fragment was prepared as follows. 200 mg of 150-kDa immunoglobulin G (IgG) (Green Cross, Korea) dissolved in 10 mM phosphate buffer was treated with 2 mg of a proteolytic enzyme, papain (Sigma) at 37°C for 2 hrs with gentle agitation. After the enzyme reaction, the immunoglobulin Fc fragment regenerated thus was subjected to chromatography for purification using sequentially a Superdex column, a protein A column and a cation exchange column. In detail, the reaction solution was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (PBS, pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. Unreacted immunoglobulin molecules (IgG) and F (ab') 2, which had a relatively high molecular weight compared to the immunoglobulin Fc fragment, were removed using their property of being eluted earlier than the Ig Fc fragment. Fab fragments having a molecular weight similar to the Ig Fc fragment were eliminated by protein A column chromatography (FIG. 1). The resulting fractions containing the Ig Fc fragment eluted from the Superdex 200 column were loaded at a flow rate of 5 ml/min onto a protein A column (Pharmacia) equilibrated with 20 mM phosphate buffer (pH 7.0), and the column was washed with the same buffer to remove proteins unbound to the column.

Then, the protein A column was eluted with 100 mM sodium citrate buffer (pH 3.0) to obtain highly pure immunoglobulin Fc fragment. The Fc fractions collected from the protein A column were finally purified using a cation exchange column (polyCAT, PolyLC Company), wherein this column loaded with the Fc fractions was eluted with a linear gradient of 0.15-0.4 M NaCl in 10 mM acetate buffer (pH 4.5), thus providing highly pure Fc fractions. The highly pure Fc fractions were analyzed by 12% SDS-PAGE (lane 2 in FIG. 2).

<Step 2> Preparation of IFN α -PEG complex

3.4-kDa polyethylene glycol having an aldehyde reactive group at both ends, ALD-PEG-ALD (Shearwater), was mixed with human interferon alpha-2b (hIFN α -2b, MW: 20 kDa) dissolved in 100 mM phosphate buffer in an amount of 5 mg/ml) at an IFN α : PEG molar ratio of 1:1, 1:2.5, 1:5, 1:10 and 1:20. To this mixture, a reducing agent, sodium cyanoborohydride (NaCNBH₃, Sigma), was added at a final concentration of 20 mM and was allowed to react at 4°C for 3 hrs with gentle agitation to allow PEG to link to the amino terminal end of interferon alpha. To obtain a 1:1 complex of PEG and interferon alpha, the reaction mixture was subjected to size exclusion chromatography using a Superdex^R column (Pharmacia). The IFN α -PEG complex was eluted from the column using 10 mM potassium phosphate buffer (pH 6.0)

as an elution buffer, and interferon alpha not linked to PEG, unreacted PEG and dimer byproducts where PEG was linked to two interferon alpha molecules were removed. The purified IFN α -PEG complex was concentrated to 5 mg/ml. 5 Through this experiment, the optimal reaction molar ratio for IFN α to PEG, providing the highest reactivity and generating the smallest amount of byproducts such as dimers, was found to be 1:2.5 to 1:5.

<Step 3> Preparation of IFN α -PEG-Fc conjugate

10 To link the IFN α -PEG complex purified in the above step 2 to the N-terminus of an immunoglobulin Fc fragment, the immunoglobulin Fc fragment (about 53 kDa) prepared in the above step 1 was dissolved in 10 mM phosphate buffer and mixed with the IFN α -PEG complex at an IFN α -PEG complex: 15 Fc molar ratio of 1:1, 1:2, 1:4 and 1:8. After the phosphate buffer concentration of the reaction solution was adjusted to 100 mM, a reducing agent, NaCNBH₃, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle 20 agitation. Through this experiment, the optimal reaction molar ratio for IFN α -PEG complex to Fc, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.

<Step 4> Isolation and purification of the IFN α -PEG-Fc

conjugate

After the reaction of the above step 3, the reaction mixture was subjected to Superdex size exclusion chromatography so as to eliminate unreacted substances and byproducts and purify the IFN α -PEG-Fc protein conjugate produced. After the reaction mixture was concentrated and loaded onto a Superdex column, 10 mM phosphate buffer (pH 7.3) was passed through the column at a flow rate of 2.5 ml/min to remove unbound Fc and unreacted substances, followed by column elution to collect IFN α -PEG-Fc protein conjugate fractions. Since the collected IFN α -PEG-Fc protein conjugate fractions contained a small amount of impurities, unreacted Fc and interferon alpha dimers, cation exchange chromatography was carried out to remove the impurities. The IFN α -PEG-Fc protein conjugate fractions were loaded onto a PolyCAT LP column (PolyLC) equilibrated with 10 mM sodium acetate (pH 4.5), and the column was eluted with a linear gradient of 0-0.5 M NaCl in 10 mM sodium acetate buffer (pH 4.5) using 1 M NaCl. Finally, the IFN α -PEG-Fc protein conjugate was purified using an anion exchange column. The IFN α -PEG-Fc protein conjugate fractions were loaded onto a PolyWAX LP column (PolyLC) equilibrated with 10 mM Tris-HCl (pH 7.5), and the column was then eluted with a linear gradient of 0-0.3 M NaCl in 10 mM Tris-HCl (pH 7.5) using 1 M NaCl, thus isolating the IFN α -PEG-Fc protein conjugate in a highly pure form.

EXAMPLE 2: Preparation II of IFN α -PEG-Fc protein conjugate

<Step 1> Preparation of Fc-PEG complex

3.4-kDa polyethylene glycol having an aldehyde reactive group at both ends, ALD-PEG-ALD (Shearwater), was 5 mixed with the immunoglobulin Fc fragment prepared in the step 1 of Example 1 at Fc: PEG molar ratios of 1:1, 1:2.5, 1:5, 1:10 and 1:20, wherein the Ig Fc fragment had been dissolved in 100 mM phosphate buffer in an amount of 15 mg/ml. To this mixture, a reducing agent, NaCNBH₃ (Sigma), 10 was added at a final concentration of 20 mM and was allowed to react at 4°C for 3 hrs with gentle agitation. To obtain a 1:1 complex of PEG and Fc, the reaction mixture was subjected to size exclusion chromatography using a Superdex[®] column (Pharmacia). The Fc-PEG complex was eluted from the 15 column using 10 mM potassium phosphate buffer (pH 6.0) as an elution buffer, and immunoglobulin Fc fragment not linked to PEG, unreacted PEG and dimer byproducts where PEG was linked to two immunoglobulin Fc fragment molecules were removed. The purified Fc-PEG complex was concentrated to 20 about 15 mg/ml. Through this experiment, the optimal reaction molar ratio for Fc to PEG, providing the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:3 to 1:10.

<Step 2> Formation and purification of conjugate of the Fc-PEG complex and interferon alpha

To link the Fc-PEG complex purified in the above step 1 to the N-terminus of IFN α , the Fc-PEG complex was mixed 5 with IFN α dissolved in 10 mM phosphate buffer at Fc-PEG complex: IFN α molar ratios of 1:1, 1:1.5, 1:3 and 1:6. After the phosphate buffer concentration of the reaction solution was adjusted to 100 mM, a reducing agent, NaCNBH₃, was added to the reaction solution at a final concentration 10 of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. After the reaction was completed, unreacted substances and byproducts were removed according to the same purification method as in the step 4 of Example 1, thus isolating the Fc-PEG-IFN α protein conjugate in a 15 highly pure form.

EXAMPLE 3: Preparation of hGH-PEG-Fc conjugate

An hGH-PEG-Fc conjugate was prepared and purified according to the same method as in Example 1, except that drug other than interferon alpha, human growth hormone 20 (hGH, MW: 22 kDa) was used and a hGH: PEG molar ratio was 1:5.

EXAMPLE 4: Preparation of (G-CSF)-PEG-Fc conjugate

A (G-CSF)-PEG-Fc conjugate was prepared and purified according to the same method as in Example 1, except that drug other than interferon alpha, human granulocyte colony stimulating factor (G-CSF), was used and an G-CSF: PEG 5 molar ratio was 1:5.

On the other hand, a ¹⁷S-G-CSF-PEG-Fc protein conjugate was prepared and purified according to the same method as described above using a G-CSF derivative, ¹⁷S-G-CSF, having a serine substitution at the seventeenth amino 10 acid residue of the native G-CSF.

EXAMPLE 5: Preparation of EPO-PEG-Fc conjugate

An EPO-PEG-Fc conjugate was prepared and purified according to the same method as in Example 1, except that drug other than interferon alpha, human erythropoietin 15 (EPO), was used and an EPO: PEG molar ratio was 1:5.

EXAMPLE 6: Preparation of protein conjugate using PEG having different reactive group

An IFN α -PEG-Fc protein conjugate was prepared using PEG having a succinimidyl propionate (SPA) reactive group 20 at both ends, as follows. 3.4-kDa polyethylene glycol, SPA-PEG-SPA (Shearwater), was mixed with 10 mg of interferon alpha dissolved in 100 mM phosphate buffer at IFN α : PEG

molar ratios of 1:1, 1:2.5, 1:5, 1:10 and 1:20. The mixture was then allowed to react at room temperature with gentle agitation for 2 hrs. To obtain a 1:1 complex of PEG and interferon alpha (IFN α -PEG complex), where PEG was linked 5 selectively to the amino group of a lysine residue of interferon alpha, the reaction mixture was subjected to Superdex size exclusion chromatography. The IFN α -PEG complex was eluted from the column using 10 mM potassium phosphate buffer (pH 6.0) as an elution buffer, and 10 interferon alpha not linked to PEG, unreacted PEG and dimer byproducts in which two interferon alpha molecules were linked to both ends of PEG were removed. To link the IFN α -PEG complex to the amino group of a lysine residue of 15 immunoglobulin Fc, the purified IFN α -PEG complex was concentrated to about 5 mg/ml, and an IFN α -PEG-Fc conjugate was prepared and purified according to the same methods as in the steps 3 and 4 of Example 1. Through this experiment, the optimal reaction molar ratio for IFN α to PEG, providing 20 the highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.5 to 1:5.

On the other hand, another IFN α -PEG-Fc conjugate was prepared according to the same methods as described above using PEG) having an N-hydroxysuccinimidyl (NHS) reactive group at both ends, NHS-PEG-NHS (Shearwater), or PEG having 25 a buthyl aldehyde reactive group at both ends, BUA-PEG-BUA (Shearwater).

EXAMPLE 7: Preparation of protein conjugate using PEG having different molecular weight

An IFN α -10K PEG complex was prepared using 10-kDa polyethylene glycol having an aldehyde reactive group at 5 both ends, ALD-PEG-ALD (Shearwater). This complex was prepared and purified according to the same method as in the step 2 of Example 1. Through this experiment, the optimal reaction molar ratio for IFN α to 10-kDa PEG, providing the highest reactivity and generating the fewest 10 byproducts such as dimers, was found to be 1:2.5 to 1:5. The purified IFN α -10K PEG complex was concentrated to about 5 mg/ml, and, using this concentrate, an IFN α -10K PEG-Fc conjugate was prepared and purified according to the same methods as in the steps 3 and 4 of Example 1.

15 EXAMPLE 8: Preparation of Fab'-S-PEG-N-Fc conjugate (-SH group)

<Step 1> Expression and Purification of Fab'

An *E. coli* transformant, BL21/poDLHF (accession number: KCCM-10511), expressing anti-tumor necrosis factor-alpha Fab', was grown in 100 ml of LB medium overnight with 20 agitation, and was inoculated in a 5-L fermentor (Marubishi) and cultured at 30°C and 500 rpm and at an air

flow rate of 20 vvm. To compensate for the insufficient nutrients for bacterial growth during fermentation, the cultures were supplemented with glucose and yeast extracts according to the fermented states of bacteria. When the 5 cultures reached an OD₆₀₀ value of 80-100, an inducer, IPTG, was added to the cultures to induce protein expression. The cultures were further cultured for 40 to 45 hrs until the OD value at 600 nm increased to 120 to 140. The fermentation fluid thus obtained was centrifuged at 10 20,000xg for 30 min. The supernatant was collected, and the pellet was discarded.

The supernatant was subjected to the following three-step column chromatography to purify anti-tumor necrosis factor-alpha Fab'. The supernatant was loaded onto a HiTrap 15 protein G column (5 ml, Pharmacia) equilibrated with 20 mM phosphate buffer (pH 7.0), and the column was eluted with 100 mM glycine (pH 3.0). The collected Fab' fractions were then loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (PBS, pH 20 7.3), and this column was eluted with the same buffer. Finally, the second Fab' fractions were loaded onto a polyCAT 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.4 M in 10 mM acetate buffer (pH 4.5), thus providing highly pure anti-tumor 25 necrosis factor-alpha Fab' fractions.

<Step 2> Preparation and purification of Fc-PEG complex

To link a PEG linker to the N-terminus of an immunoglobulin Fc, the immunoglobulin Fc prepared according to the same method as in the step 1 of Example 1 was dissolved in 100 mM phosphate buffer (pH 6.0) at a concentration of 5 mg/ml, and was mixed with NHS-PEG-MAL (3.4 kDa, Shearwater) at an Fc: PEG molar ratio of 1:10, followed by incubation at 4°C for 12 hrs with gentle agitation.

After the reaction was completed, the reaction buffer was exchanged with 20 mM sodium phosphate buffer (pH 6.0) to remove unbound NHS-PEG-MAL. Then, the reaction mixture was loaded onto a polyCAT column (PolyLC). The column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 6.0). During this elution, the immunoglobulin Fc-PEG complex was eluted earlier than unreacted immunoglobulin Fc, and the unreacted Ig Fc was eluted later, thereby eliminating the unreacted Ig Fc molecules.

<Step 3> Preparation and purification of Fab'-S-PEG-N-Fc conjugate (-SH group)

To link the immunoglobulin Fc-PEG complex to a cysteine group of the Fab', the Fab' purified in the above step 1 was dissolved in 100 mM sodium phosphate buffer (pH 7.3) at a concentration of 2 mg/ml, and was mixed with the

immunoglobulin Fc-PEG complex prepared in the above step 2 at a Fab': complex molar ratio of 1:5. The reaction mixture was concentrated to a final protein concentration of 50 mg/ml and incubated at 4°C for 24 hrs with gentle agitation.

5 After the reaction was completed, the reaction mixture was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. The coupled Fab'-S-PEG-N-Fc conjugate was 10 eluted relatively earlier due to its high molecular weight, and unreacted immunoglobulin Fc-PEG complex and Fab' were eluted later, thereby eliminating the unreacted molecules. To completely eliminate unreacted immunoglobulin Fc-PEG, the collected Fab'-S-PEG-N-Fc conjugate fractions were 15 again loaded onto a polyCAT 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 6.0), thus providing a pure Fab'-S-PEG-N-Fc conjugate comprising the Fc-PEG complex linked to an -SH group near the C-terminus 20 of the Fab'.

EXAMPLE 9: Preparation of Fab'-N-PEG-N-Fc conjugate (N-terminus)

<Step 1> Preparation and purification of Fab'-PEG complex (N-terminus)

40 mg of the Fab' purified in the step 1 of Example 8 was dissolved in 100 mM sodium phosphate buffer (pH 6.0) at a concentration of 5 mg/ml, and was mixed with butyl ALD-PEG-butyl ALD (3.4 kDa, Nektar) at a Fab': PEG molar ratio 5 of 1:5. A reducing agent, NaCNBH₃, was added to the reaction mixture at a final concentration of 20 mM, and the reaction mixture was then allowed to react at 4°C for 2 hrs with gentle agitation.

After the reaction was completed, the reaction buffer 10 was exchanged with 20 mM sodium phosphate buffer (pH 6.0). Then, the reaction mixture was loaded onto a polyCAT column (PolyLC). The column was eluted with a linear NaCl gradient of 0.15-0.4 M in 20 mM acetate buffer (pH 4.5). During this column elution, the Fab'-PEG complex comprising the PEG 15 linker linked to the N-terminus of the Fab' was eluted earlier than unreacted Fab', and the unreacted Fab' was eluted later, thereby eliminating the unreacted Fab' molecules.

<Step 2> Preparation and purification of Fab'-N-PEG-N-Fc 20 conjugate

To link the Fab'-PEG complex purified in the above step 1 to the N-terminus of an immunoglobulin Fc, the Fab'-PEG complex was dissolved in 100 mM sodium phosphate buffer (pH 6.0) at a concentration of 10 mg/ml, and was mixed with 25 the immunoglobulin Fc dissolved in the same buffer at a

Fab'-PEG complex: Fc molar ratio of 1:5. After the reaction mixture was concentrated to a final protein concentration of 50 mg/ml, a reducing agent, NaCNBH₃, was added to the reaction mixture at a final concentration of 20 mM, and the 5 reaction mixture was then reacted at 4°C for 24 hrs with gentle agitation.

After the reaction was completed, the reaction mixture was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), 10 and the column was eluted with the same buffer at a flow rate of 1 ml/min. The coupled Fab'-N-PEG-N-Fc conjugate was eluted relatively earlier due to its high molecular weight, and unreacted immunoglobulin Fc and Fab'-PEG complex were eluted later, thereby eliminating the unreacted molecules. 15 To completely eliminate the unreacted immunoglobulin Fc molecules, the collected Fab'-N-PEG-N-Fc conjugate fractions were again loaded onto a polyCAT 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 6.0), thus providing a pure Fab'-N-PEG-N-Fc conjugate 20 comprising the immunoglobulin Fc-PEG complex linked to the N-terminus of the Fab'.

EXAMPLE 10: Preparation and purification of deglycosylated immunoglobulin Fc

200 mg of an immunoglobulin Fc prepared according to the same method as in Example 1 was dissolved in 100 mM phosphate buffer (pH 7.5) at a concentration of 2 mg/ml, and was mixed with 300 U/mg of a deglycosylase, PNGase F (NEB). The reaction mixture was allowed to react at 37°C for 24 hrs with gentle agitation. Then, to purify the deglycosylated immunoglobulin Fc, the reaction mixture was loaded onto a SP Sepharose FF column (Pharmacia), and the column was eluted with a linear NaCl gradient of 0.1-0.6 M in 10 mM acetate buffer (pH 4.5) using 1 M NaCl. The native immunoglobulin Fc was eluted earlier, and the deglycosylated immunoglobulin Fc (DG Fc) was eluted later.

EXAMPLE 11: Preparation of IFN α -PEG-DG Fc conjugate

To link the deglycosylated immunoglobulin Fc prepared in Example 10 to the IFN α -PEG complex purified in the step 15 2 of Example 1, the IFN α -PEG complex was mixed with the DG Fc dissolved in 10 mM phosphate buffer at IFN α -PEG complex: DG Fc molar ratios of 1:1, 1:2, 1:4 and 1:8. After the phosphate buffer concentration of the reaction solution was 20 adjusted to 100 mM, a reducing agent, NaCNBH₃, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. Through this experiment, the optimal reaction 20 molar ratio for IFN α -PEG complex to DG Fc, providing the

highest reactivity and generating the fewest byproducts such as dimers, was found to be 1:2.

After the coupling reaction, the reaction mixture was subjected to size exclusion chromatography using a Superdex^R column (Pharmacia) so as to eliminate unreacted substances and byproducts and purify the IFN α -PEG-DG Fc protein conjugate. After the reaction mixture was loaded onto the column, a phosphate buffer (pH 7.3) was passed through the column at a flow rate of 2.5 ml/min to remove unbound DG Fc and unreacted substances, followed by column elution to collect IFN α -PEG-DG Fc protein conjugate fractions. Since the collected IFN α -PEG-DG Fc protein conjugate fractions contained a small amount of impurities, unreacted DG Fc and IFN α -PEG complex, and cation exchange chromatography was carried out to remove the impurities. The IFN α -PEG-DG Fc protein conjugate fractions were loaded onto a PolyCAT LP column (PolyLC) equilibrated with 10 mM sodium acetate (pH 4.5), and the column was eluted with a linear gradient of 0-0.6 M NaCl in 10 mM sodium acetate buffer (pH 4.5) using 1 M NaCl. Finally, the IFN α -PEG-DG Fc protein conjugate was purified using an anion exchange column. The IFN α -PEG-Fc protein conjugate fractions were loaded onto a PolyWAX LP column (PolyLC) equilibrated with 10 mM Tris-HCl (pH 7.5), and the column was then eluted with a linear gradient of 0-0.3 M NaCl in 10 mM Tris-HCl (pH 7.5) using 1 M NaCl, thus isolating the IFN α -PEG-DG Fc protein conjugate in a highly

pure form.

EXAMPLE 12: Preparation and purification of recombinant
aglycosylated immunoglobulin Fc derivative

<Preparation of IgG4 Fc derivative 1 expression vector>

5 To prepare human immunoglobulin IgG4 heavy chain constant regions, a first derivative (IgG4 delta-Cys), having a nine amino acid deletion at the amino terminus of the native hinge region, and a second derivative (IgG4 monomer), lacking the hinge region by a deletion of all of
10 twelve amino acids of the hinge region, were prepared. As an expression vector containing an *E. coli* secretory sequence, pt14S1SH-4T20V22Q (Korean Pat. No. 38061), developed prior to the present invention by the present invention, was used.

15 To obtain human immunoglobulin IgG4 heavy chain constant regions, RT-PCR was carried out using RNA isolated from human blood cells as a template, as follows. First, total RNA was isolated from about 6 ml of blood using a Qiamp RNA blood kit (Qiagen), and gene amplification was
20 performed using the total RNA as a template and a One-Step RT-PCR kit (Qiagen). In this PCR, a pair of synthesized primers represented by SEQ ID Nos. 1 and 2 and another pair of synthesized primers represented by SEQ ID Nos. 2 and 3 were used. SEQ ID NO. 1 is a nucleotide sequence starting

from the 10th residue, serine, of 12 amino acid residues, below, of the hinge region of IgG4. SEQ ID NO. 3 was designed to have a nucleotide sequence encoding a C_H2 domain having alanine as a first amino acid residue. SEQ ID NO. 2 5 was designed to have a BamHI recognition site containing a stop codon.

	1	2	3	4	5	6	7	8	9	10	11	12	
	gag	tcc	aaa	tat	ggt	ccc	cca	tgc	cca	tca	tgc	cca	
	ctc	agg	ttt	ata	cca	ggg	ggt	acg	ggt	agt	acg	ggt	
10		Glu	Ser	Lys	Tyr	Gly	Pro	Pro	Cys	Pro	Ser	Cys	Pro

To clone each of the amplified IgG4 constant region fragments into an expression vector containing an *E. coli* secretory sequence derivative, the pT14S1SH-4T20V22Q (Korean Pat. No. 38061) developed prior to the present 15 invention by the present inventors was used. This expression vector contains a heat-stable enterotoxin secretory sequence derivative that has a nucleotide sequence represented by SEQ ID NO. 4. To facilitate cloning, a StuI recognition site was inserted into an end 20 of the *E. coli* heat-stable enterotoxin secretory sequence derivative of the pT14S1SH-4T20V22Q plasmid through site-directed mutagenesis using a pair of primers represented by SEQ ID Nos. 5 and 6 to induce mutagenesis to introduce the StuI site at a nucleotide sequence coding for the last

amino acid residue of the secretory sequence. This insertion of the *StuI* site was found to be successful by DNA sequencing. The resulting pT14S1SH-4T20V22Q plasmid containing a *StuI* site was designated as pmSTII. The pmSTII plasmid was treated with *StuI* and *BamHI* and subjected to agarose gel electrophoresis, and a large fragment (4.7 kb), which contained the *E. coli* heat-stable enterotoxin secretory sequence derivative, was purified. Then, the amplified gene fragments were digested with *BamHI* and 10 ligated with the linearized expression vector, thus providing pSTIIdCG4Fc and pSTIIG4Mo.

The final expression vectors were individually transformed into *E. coli* BL21(DE3), and the resulting transformants were designated as BL21/pSTIIdCG4Fc (HM10932) 15 and BL21/pSTIIdCG4Mo (HM10933), which were deposited at the Korean Culture Center of Microorganisms (KCCM) on Sep. 15, 2004 and assigned accession numbers KCCM-10597 and KCCM-10598, respectively. Thereafter, when the cultures reached 20 an OD₆₀₀ value of 80, an inducer, IPTG, was added to the cultures to induce protein expression. The cultures were further cultured for 40 to 45 hrs until the OD value at 600 nm increased to 100 to 120. The *E. coli* cells collected from the fermentation fluids were disrupted, and the resulting cell lysates were subjected to two-step column 25 chromatography to purify the recombinant immunoglobulin constant region derivatives present in the cytosol of *E.*

coli.

5 5 ml of a protein-A affinity column (Pharmacia) was equilibrated with PBS, and the cell lysates were loaded onto the column at a flow rate of 5 ml/min. Unbound proteins were washed out with PBS, and bound proteins were eluted with 100 mM citrate (pH 3.0). The collected fractions were desalting using a HiPrep 26/10 desalting column (Pharmacia) with 10 mM Tris buffer (pH 8.0). Then, secondary anion exchange column chromatography was carried 10 out using 50 ml of a Q HP 26/10 column (Pharmacia). The primary purified recombinant aglycosylated immunoglobulin Fc fractions were loaded onto the Q-Sepharose HP 26/10 column, and the column was eluted with a linear gradient of 0-0.2 M NaCl in 10 mM Tris buffer (pH 8.0), thus providing 15 a highly pure recombinant aglycosylated immunoglobulin Fc (AG Fc) derivative, IgG4 delta-Cys and a highly pure IgG4 monomer fraction.

EXAMPLE 13: Preparation of conjugate of IFN α -PEG complex and recombinant AG Fc derivative

20 According to the same methods as in Examples 1 and 11, the IFN α -PEG complex was linked to the N terminus of the IgG4 delta-Cys as an AG Fc derivative prepared in Example 12. After the coupling reaction, unreacted substances and byproducts were removed from the reaction

mixture, and the thus-produced IFN α -PEG-AG Fc protein conjugate (I) was primarily purified using 50 ml of a Q HP 26/10 column (Pharmacia) and further purified by a high-pressure liquid chromatographic assay using a polyCAT 21.5 \times 250 column (polyLC), thus purifying the conjugate to a high degree. The coupling reaction solution was desalted using a HiPrep 26/10 desalting column (Pharmacia) with 10 mM Tris buffer (pH 8.0). Then, the reaction solution was then loaded onto 50 ml of a Q HP 26/10 column (Pharmacia) at a flow rate of 8 ml/min, and this column was eluted with a linear NaCl gradient of 0-0.2 M to obtain desired fractions. The collected fractions were again loaded onto a polyCAT 21.5 \times 250 column equilibrated with 10 mM acetate buffer (pH 5.2) at a flow rate of 15 ml/min, and this column was eluted with a linear NaCl gradient of 0.1-0.3 M, thus providing highly pure fractions. According to the same method as described above, another IFN α -PEG-AG Fc protein conjugate (II) was prepared using another AG Fc derivative prepared in Example 12, IgG4 monomer.

EXAMPLE 14: Preparation of EPO-PEG-recombinant AG Fc derivative conjugate

According to the same method as in Example 13, an EPO-PEG-recombinant AG Fc derivative conjugate was prepared by linking an AG Fc derivative, IgG4 delta-Cys, to the EPO-

PEG complex.

COMPARATIVE EXAMPLE 1: Preparation of IFN α -40K PEG complex

5 mg of interferon alpha was dissolved in 100 mM phosphate buffer to obtain a final volume of 5 ml, and was
5 mixed with 40-kDa activated methoxy-PEG-aldehyde (Shearwater), at an IFN α :40-kDa PEG molar ratio of 1:4. To this mixture, a reducing agent, NaCNBH₃ was added at a final concentration of 20 mM and was allowed to react at 4°C for 18 hrs with gentle agitation. To inactivate PEG, which did
10 not react with IFN α , Ethanolamine was added to the reaction mixture at a final concentration of 50mM.

A Sephadex G-25 column (Pharmacia) was used to remove unreacted PEG and exchange the buffer with another buffer. First, this column was equilibrated with two column volumes (CV) of 10 mM Tris-HCl buffer (pH 7.5), and was loaded with the reaction mixture. Flow throughs were detected by measuring the absorbance at 260 nm using a UV spectrophotometer. When the column was eluted with the same buffer, interferon alpha modified by adding PEG having a higher molecular weight to its N-terminus was eluted earlier, and unreacted PEG was eluted later, thus allowing isolation of only IFN α -40K PEG.
20

The following chromatography was carried out to further purify the IFN α -40K PEG complex from the collected

fractions. 3 ml of a PolyWAX LP column (PolyLC) was equilibrated with 10 mM Tris-HCl (pH 7.5). The collected fractions containing the IFN α -40K PEG complex was loaded onto the column at a flow rate of 1 ml/min, and the column 5 was washed with 15 ml of the equilibrium buffer. Then, the column was eluted with a linear NaCl gradient of 0-100% using 30 ml of 1 M NaCl, thus eluting interferon alpha conjugated to tri-, di- and mono-PEG, sequentially. To further purify the mono-PEG-conjugated interferon alpha, 10 the collected fractions containing the mono-PEG-conjugated interferon alpha were subjected to size exclusion chromatography. The fractions were concentrated and loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.0), and the column was 15 eluted with the same buffer at a flow rate of 1 ml/min. The tri- and di-PEG-conjugated interferon alpha molecules were removed based on their property of being eluted earlier than the mono-PEG-conjugated interferon alpha, thus isolating the mono-PEG-conjugated interferon alpha in a 20 highly pure form.

According to the same method as described above, 40-kDa PEG was conjugated to the N-terminus of human growth hormone, granulocyte colony stimulating factor (G-CSF), and a derivative of G-CSF, thus providing hGH-40K PEG, G-CSF-25 40K PEG and 40K PEG-¹⁷S-G-CSF derivative complexes.

COMPARATIVE EXAMPLE 2: Preparation of IFN α -PEG-albumin conjugate

To link the IFN α -PEG complex purified in the step 2 of Example 1 to the N-terminus of albumin, the IFN α -PEG complex was mixed with human serum albumin (HSA, about 67 kDa, Green Cross) dissolved in 10 mM phosphate buffer at an IFN α -PEG complex: albumin molar ratio of 1:1, 1:2, 1:4 and 1:8. After the phosphate buffer concentration of the reaction solution was adjusted to 100 mM, a reducing agent, 10 NaCNBH₃, was added to the reaction solution at a final concentration of 20 mM and was allowed to react at 4°C for 20 hrs with gentle agitation. Through this experiment, the optimal reaction molar ratio for IFN α -PEG complex to albumin, providing the highest reactivity and generating 15 the fewest byproducts such as dimers, was found to be 1:2.

After the coupling reaction, the reaction mixture was subjected to size exclusion chromatography using a Superdex[®] column (Pharmacia) so as to eliminate unreacted substances and byproducts and purify the IFN α -PEG-albumin protein conjugate produced. After the reaction mixture was concentrated and loaded onto the column, 10 mM sodium acetate buffer passed through the column at a flow rate of 2.5 ml/min to remove unbound albumin and unreacted substances, followed by column elution to purify only IFN α -PEG-albumin protein conjugate. Since the collected IFN α -

PEG-albumin protein conjugate fractions contained a small amount of impurities, unreacted albumin and interferon alpha dimers, cation exchange chromatography was carried out to remove the impurities. The IFN α -PEG-albumin protein conjugate fractions were loaded onto a SP5PW column (Waters) equilibrated with 10 mM sodium acetate (pH 4.5), and the column was eluted with a linear gradient of 0-0.5 M NaCl in 10 mM sodium acetate buffer (pH 4.5) using 1 M NaCl, thus isolating the IFN α -PEG-albumin protein conjugate in a highly pure form.

According to the same method as described above, albumin was conjugated to human growth hormone, G-CSF, and a derivative of G-CSF, thus providing hGH-PEG-albumin, G-CSF-PEG-albumin and 17 S-G-CSF-PEG-albumin conjugates.

15 COMPARATIVE EXAMPLE 3: Preparation of Fab'-S-40K PEG complex

The free cysteine residue of the Fab' purified in the step 1 of Example 8 was activated by incubation in an activation buffer (20 mM sodium acetate (pH 4.0), 0.2 mM DTT) for 1 hr. After the buffer was exchanged by a PEG modification buffer, 50 mM potassium phosphate (pH 6.5), maleimide-PEG (MW: 40 kDa, Shearwater) was added thereto at a Fab':40-kDa PEG molar ratio of 1:10 and was reacted to react at 4°C for 24 hrs with gentle agitation.

After the reaction was completed, the reaction solution was loaded onto a Superdex 200 column (Pharmacia) equilibrated with 10 mM sodium phosphate buffer (pH 7.3), and the column was eluted with the same buffer at a flow rate of 1 ml/min. The Fab' conjugated 40-kDa PEG (Fab'-40K PEG) was eluted relatively earlier due to its high molecular weight, and unreacted Fab' was eluted later, thereby eliminating the unreacted Fab'. To completely eliminate the unreacted Fab', the collected Fab'-40K PEG complex fractions were again loaded onto a polyCAT 21×250 column (PolyLC), and this column was eluted with a linear NaCl gradient of 0.15-0.5 M in 20 mM sodium phosphate buffer (pH 4.5), thus providing a pure Fab'-S-40K PEG complex comprising 40-kDa PEG linked to an -SH group of the Fab'.

EXPERIMENTAL EXAMPLE 1: Identification and quantitative analysis of the protein conjugates

<1-1> Identification of the protein conjugates

The protein conjugates prepared in the above Examples were analyzed by non-reduced SDS-PAGE using a 4-20% gradient gel and a 12% gel and ELISA (R&D System).

As a result of SDS-PAGE analysis, as shown in FIG. 3, a coupling reaction of a physiological polypeptide, a non-peptide polymer, PEG, and an immunoglobulin Fc fragment

resulted in the successful production of an IFN α -PEG-Fc conjugate (A), a ^{17}S er-G-CSF-PEG-Fc conjugate (B) and an EPO-PEG-Fc conjugate (C).

In addition, the DG Fc prepared in Example 10 was
5 analyzed by non-reduced 12% SDS-PAGE. As shown in FIG. 6b,
a DG Fc band was detected at a position, which corresponds
to the molecular weight of the native Fc lacking sugar
moieties.

<1-2> Quantitative analysis of the protein conjugates

10 The protein conjugates prepared in the above Examples
were quantified by size exclusion chromatography using a
HiLoad 26/60 Superdex 75 column (Pharmacia) and 10 mM
potassium phosphate buffer (pH 6.0) as an elution buffer,
wherein a peak area of each protein conjugate was compared
15 to that of a control group. Previously quantitatively
analyzed standards, IFN α , hGH, G-CSF, ^{17}S -G-CSF, EPO and Fc,
were individually subjected to size exclusion
chromatography, and a conversion factor between a
concentration and a peak was determined. A predetermined
20 amount of each protein conjugate was subjected to the same
size exclusion chromatography. By subtracting a peak area
corresponding to an immunoglobulin Fc fragment from the
thus-obtained peak area, a quantitative value for a
physiologically active protein present in each protein
25 conjugate was determined. FIG. 4 shows the result of size

exclusion chromatography of the purified IFN α -PEG-Fc conjugate, wherein a single peak was observed. This result indicates that the purified protein conjugate does not contain multimeric impurities such as a dimer, a trimer or 5 a higher number of monomers.

When a physiologically active polypeptide conjugated to Fc was quantitatively analyzed using an antibody specific to the physiologically active polypeptide, the antibody was prevented from binding to the polypeptide, 10 resulting in a value lower than an actual value calculated by the chromatography. In the case of the IFN α -PEG-Fc conjugate, an ELISA resulted in an ELISA value corresponding to about 30% of an actual value.

15 <1-3> Evaluation of purity and mass of the protein conjugates

The protein conjugates prepared in the above Examples were subjected to size exclusion chromatography, and absorbance was measured at 280 nm. As a result, the IFN α -PEG-Fc, hGH-PEG-Fc, G-CSF-PEG-Fc and ^{17}Ser -G-CSF-PEG-Fc 20 conjugates displayed a single peak at the retention time of a 70 to 80-kDa substance.

On the other hand, reverse phase HPLC was carried out to determine purities of the protein conjugates prepared in Examples 1, 11 and 13, IFN α -PEG-Fc, IFN α -PEG-DG Fc and 25 IFN α -PEG-recombinant AG Fc derivative. A reverse phase

column (259 VHP54 column, Vydac) was used. The column was eluted with a 40-100% acetonitrile gradient with 0.5% TFA, and purities were analyzed by measuring absorbance at 280 nm. As a result, as shown in FIG. 8, the samples contain no 5 unbound interferon or immunoglobulin Fc, and all of the protein conjugates, IFN α -PEG-Fc (A), IFN α -PEG-DG Fc (B) and IFN α -PEG-recombinant AG Fc derivative (C), were found to have purity greater than 96%.

To determine accurate molecular weights of the 10 purified protein conjugates, mass for each conjugate was analyzed using a high-throughput MALDI-TOF mass spectrophotometer (Voyager DE-STR, Applied Biosystems). Sinapinic acid was used as a matrix. 0.5 μ l of each test sample was coated onto a sample slide and air-dried, again 15 mixed with the equal volume of a matrix solution and air-dried, and introduced into an ion source. Detection was carried out in a positive fashion using a linear mode TOF analyzer. Ions were accelerated with a split extraction source operated with delayed extraction (DE) using a 20 delayed extraction time of 750 nsec to 1500 nsec at a total acceleration voltage of about 2.5 kV.

The molecular weights observed by MALDI-TOF mass spectrometry for the Fc protein conjugates prepared in Examples are given in Table 1, below. FIG. 5 shows the 25 result of MALDI-TOF mass spectrometry of the EPO-PEG-Fc conjugate, and FIG. 7 shows the results of MALDI-TOF mass

spectrometry of the IFN α -PEG-Fc and IFN α -PEG-DG Fc conjugates. As a result, the EPO-PEG-Fc protein conjugate was found to have a purity of more than 95% and a molecular weight very close to a theoretical MW. Also, EPO was found 5 to couple to the immunoglobulin Fc fragment at a ratio of 1:1.

TABLE 1

	Theoretical MW (kDa)	Measured MW (kDa)
IFN α -PEG-Fc (E.1)	75.4	75.9
hGH-PEG-Fc (E.3)	78.4	78.6
G-CSF-PEG-Fc (E.4)	75.3	75.9
¹⁷ S-G-CSF derivative-PEG-Fc (E.4)	75.0	75.9
EPO-PEG-Fc (E.5)	91.4	91.0

In addition, when the Fc and DG Fc prepared in Example 10 were examined for their molecular weights by 10 MALDI-TOF mass spectrometry, the DG Fc was found to be 50 kDa, which is about 3-kDa less than native Fc (FIG. 6a). Since the 3-kDa MW corresponds to the theoretical size of sugar moieties, the results demonstrate that the sugar 15 moieties are completely removed.

Table 2, below, shows the results of MALDI-TOF mass spectrometry of the IFN α -PEG-DG Fc conjugate prepared in Example 11 and the IFN α -PEG-recombinant AG Fc derivative conjugates (I and II) prepared in Example 13. The IFN α -PEG-DG Fc conjugate was found to be 3 kDa lighter, and the 20 IFN α -PEG-recombinant AG Fc derivative conjugate (I) to be

about 3-4 kDa lighter, than the IFN α -PEG-Fc conjugate of 75.9 kDa. The IFN α -PEG-recombinant AG Fc derivative conjugate (II) coupled to an Fc monomer showed a molecular weight decreased by 24.5 kDa corresponding to the molecular weight of the Fc monomer.

TABLE 2

	Theoretical MW (kDa)	Measured MW (kDa)
IFN α -PEG-DG Fc (E.11)	72.8	73.0
IFN α -PEG-recombinant AG Fc derivative (I) (E.13)	72.3	72.2
IFN α -PEG-recombinant AG Fc derivative (II) (E.13)	46.8	46.6

EXPERIMENTAL EXAMPLE 2: Pharmacokinetic analysis I

Native forms of physiologically active proteins (controls) and the protein conjugates prepared in Examples 10 and Comparative Examples, -40K PEG complexes, -PEG-albumin conjugates, -PEG-Fc conjugates, -PEG-DG Fc conjugates and -PEG-recombinant AG Fc derivative conjugates, were evaluated for serum stability and pharmacokinetic parameters in SD rats (five rats per group). The controls, and the -40K PEG 15 complexes, -PEG-albumin conjugates, -PEG-Fc conjugates, -PEG-DG Fc conjugates and -PEG-recombinant AG Fc derivative conjugates (test groups) were individually injected subcutaneously at a dose of 100 μ g/kg. After the subcutaneous injection, blood samples were collected at

0.5, 1, 2, 4, 6, 12, 24, 30, 48, 72 and 96 hrs in the control groups, and, in the test groups, at 1, 6, 12, 24, 30, 48, 72, 96, 120, 240 and 288 hrs. The blood samples were collected in tubes with an anticoagulant, heparin, and

5 centrifuged for 5 min using an Eppendorf high-speed micro centrifugator to remove blood cells. Serum protein levels were measured by ELISA using antibodies specific to the physiologically active proteins.

The results of pharmacokinetic analyses of the native forms of IFN α , hGH, G-CSF and EPO, and -40K PEG complexes thereof, -PEG-albumin conjugates thereof, -PEG-Fc conjugates thereof and -PEG-DG Fc conjugates thereof, are given in Tables 3 to 7, below. In the following tables, T_{max} indicates the time taken to reach the maximal drug serum concentration, $T_{1/2}$ indicates the serum half-life of a drug, and MRT (mean residence time) indicates the mean time that a drug molecule resides in the body.

TABLE 3
Pharmacokinetics of interferon alpha

	Native IFN α	IFN α -40K PEG (C.E.1)	IFN α -PEG-albumin (C.E.2)	IFN α -PEG-Fc (E.1)	IFN α -PEG-DG Fc (E.11)	IFN α -PEG-recombinant AG Fc derivative (I) (E.13)	IFN α -PEG-recombinant AG Fc derivative (II) (E.13)
T_{max} (hr)	1.0	30	12	30	48	24	24
$T_{1/2}$ (hr)	1.7	35.8	17.1	90.4	71.0	61.2	31.2
MRT (hr)	2.1	71.5	32.5	150.1	120.6	111.0	58.8

TABLE 4
Pharmacokinetics of human growth factor

	Native hGH	hGH-40K PEG (C.E.1)	hGH-PEG-albumin (C.E.2)	hGH-PEG-Fc (E.3)
T _{max} (hr)	1.0	12	12	12
T _{1/2} (hr)	1.1	7.7	5.9	11.8
MRT (hr)	2.1	18.2	13.0	18.8

TABLE 5
Pharmacokinetics of G-CSF

	Native G-CSF	G-CSF-40K PEG (C.E.1)	G-CSF-PEG-albumin (C.E.2)	G-CSF-PEG-Fc (E.4)
T _{max} (hr)	2.0	12	12	12
T _{1/2} (hr)	2.8	4.8	5.2	6.9
MRT (hr)	5.2	24.5	25.0	32.6

5

TABLE 6
Pharmacokinetics of ¹⁷S-G-CSF derivative

	Native ¹⁷ S-G-CSF derivative	¹⁷ S-G-CSF-40K PEG (C.E.1)	¹⁷ S-G-CSF-PEG-albumin (C.E.2)	¹⁷ S-G-CSF-PEG-Fc (E.4)
T _{max} (hr)	2.0	24	24	24
T _{1/2} (hr)	2.9	4.3	6.4	7.0
MRT (hr)	5.8	24.4	25.1	33.2

TABLE 7
Pharmacokinetics of EPO

	Native EPO	Highly glycosylated EPO	EPO-PEG-Fc (E.5)	EPO-PEG-recombinant AG Fc derivative (E.13)
T _{max} (hr)	6.0	12	30	48
T _{1/2} (hr)	9.4	18.4	61.5	87.9

MRT (hr)	21.7	26.8	117.6	141.6
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As shown from the data of Table 3 and the pharmacokinetic graph of FIG. 9, the IFN α -PEG-Fc protein conjugate had a serum half-life of 90.4 hrs, which was about 50 times higher than that of native IFN α and about 5 2.5 times higher than that of IFN α -40K PEG having a half-life of 35.8 hrs, prepared in Comparative Example 1. Also, the IFN α -PEG-Fc protein conjugate of the present invention was found to be superior in serum half-life to IFN α -PEG-albumin, which has a half-life of 17.1 hrs.

10 On the other hand, as shown in Table 3 and FIG. 11, the IFN α -PEG-DG Fc conjugate had a serum half-life of 71.0 hrs, which was almost the same as the IFN α -PEG-Fc conjugate, indicating that the deglycosylation of Fc does not greatly affect the in vivo stability of the IFN α -PEG-DG 15 Fc conjugate. Also, the conjugate prepared using the recombinant AG Fc derivative produced by a recombinant method was found to have an effect identical to that of the native form-derived DG Fc. However, the serum half-life of a complex coupled to an Fc monomer was about half that of a 20 complex coupled to a normal Fc dimer.

As shown in Table 4, human growth hormone also showed an extended serum half-life when conjugated to the IgG Fc fragment according to the present invention. That is, compared to the native form (1.1 hrs), the hGH-40K PEG 25 complex and hGH-PEG-albumin conjugate had slightly

increased half-lives of 7.7 hrs and 5.9 hrs, respectively, whereas the hGH-PEG-Fc protein conjugate of the present invention displayed a greatly extended serum half-life of 11.8 hrs.

5 As apparent from the pharmacokinetic data of G-CSF and its derivative in Table 5 and 6, the G-CSF-PEG-Fc and ¹⁷S-G-CSF-PEG-Fc conjugates displayed a much longer serum half-life than the -40K PEG complex and -PEG-albumin conjugate. The immunoglobulin Fc fragment was found in the
10 serum to prolong the duration of action of physiologically active proteins in native forms, as well as in their derivatives having alterations of certain amino acid residues in similar levels to the native forms. From these results, it is easily predictable that the method of the
15 present invention will have a similar effect on other proteins and their derivatives.

As shown in Table 7 and FIG. 10, the conjugation of the native glycosylated EPO to the Fc fragment also resulted in an increase in serum half-life. That is, EPO
20 had a serum half-life of 9.4 hrs in the native form, and a prolonged serum half-life of 18.4 hrs when highly glycosylated to improve serum stability. The EPO-PEG-Fc conjugate, comprising EPO coupled to the immunoglobulin Fc fragment according to the present invention, displayed a
25 markedly prolonged serum half-life of 61.5 hrs. Also, when conjugated to the *E. coli*-derived recombinant aglycosylated

(AG) Fc derivative, the half-life of EPO increased to 87.9 hrs, indicating that the aglycosylation of the Fc fragment allows the preparation of a protein conjugate not affecting serum stability of the protein without antibody functions.

5 As apparent from the above results, the protein conjugates covalent-bonded to the immunoglobulin Fc fragment through a non-peptide polymer according to the present invention displayed serum half-lives increased several to several tens to that of the native form. Also,
10 when the immunoglobulin Fc was aglycosylated by production in *E. coli* or deglycosylated by enzyme treatment, its effect of increasing the serum half-life of its protein conjugate was maintained at a similar level.

15 In particular, compared to proteins modified with 40-kDa PEG having the longest duration of action among PEG molecules for increasing the duration of action of proteins in the serum, the immunoglobulin Fc protein conjugates had much superior serum stability. In addition, compared to protein conjugates coupled to albumin instead of the
20 immunoglobulin Fc, the protein conjugates of the present invention displayed excellent serum stability, indicating that the protein conjugates of the present invention are effective in developing long-acting forms of protein drugs. These results, that the present protein conjugates have
25 excellent effects on serum stability and MRT in a broad range of proteins including colony stimulating factor

derivatives by point mutation compared to conventional PEG- or albumin-conjugated proteins, indicate that the stability and duration-extending effects of the present protein conjugates are applicable to other physiologically active 5 polypeptides.

On the other hand, when the IFNa-10K PEG-Fc protein conjugate (Example 7) prepared using a non-peptide polymer, 10-kDa PEG, was evaluated for its serum half-life according to the same method as described above, it showed a serum 10 half-life of 48.8 hrs, which was somewhat shorter than the serum half-life (79.7 hrs) of a protein conjugate prepared using 3.4-kDa PEG.

In addition, the serum half-lives of the protein conjugates decrease with increasing molecular weight of the 15 non-peptide polymer PEG. These results indicate that the major factor increasing the serum stability and duration of the protein conjugates is the conjugated immunoglobulin Fc fragment rather than the non-peptide polymer.

Even when the reactive group of PEG was exchanged 20 with a reactive group other than the aldehyde group, protein conjugates with the PEG showed similar patterns in apparent molecular weight and serum half-life to those coupled to PEG having an aldehyde reactive group.

EXPERIMENTAL EXAMPLE 3: Pharmacokinetic analysis II

To determine the serum half-lives of the Fab'-S-PEG-N-Fc and Fab'-N-PEG-N-Fc conjugates prepared in Example 8 and 9 and the Fab'-S-40K PEG complex prepared in Comparative Example 3, drug pharmacokinetic analysis was 5 carried out according to the same method as in Experimental Example 2 using Fab' as a control, the conjugates and the complex. The results are given in FIG. 12.

As shown in FIG. 12, the Fab'-S-PEG-N-Fc and Fab'-N-PEG-N-Fc conjugates displayed a serum half-life prolonged 10 two or three times compared to the Fab' or Fab'-S-40K PEG complex.

EXPERIMENTAL EXAMPLE 4: Evaluation of intracellular activity of the protein conjugates

<4-1> Comparison of the IFN α protein conjugates for 15 intracellular activity

To compare the intracellular activity of the IFN α protein conjugates, the IFN α -PEG-Fc (Example 1), IFN α -PEG-DG Fc (Example 11), IFN α -PEG-recombinant AG Fc derivative (Example 13), IFN α -40K PEG (Comparative Example 1) and 20 IFN α -PEG-albumin (Comparative Example 2) were evaluated for antiviral activity by a cell culture bioassay using Madin Darby Bovine Kidney (MDBK) cells (ATCC CCL-22) infected with vesicular stomatitis virus. Nonpegylated interferon alpha-2b, available from the National Institute for

Biological Standards and Controls (NIBSC), was used as a standard material.

MDBK cells were cultured in MEM (minimum essential medium, JBI) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO₂ condition. Samples to be analyzed and the standard material were diluted with the culture medium to predetermined concentrations, and 100-μl aliquots were placed onto each well of a 96-well plate. The cultured cells were detached, added to the plate containing the samples in a volume of 100 μl, and cultured for about 1 hr at 37°C under 5% CO₂ condition. Then, 50 μl of vesicular stomatitis virus (VSV) of 5-7×10³ PFU was added to each well of the plate, and the cells were further cultured for about 16 to 20 hrs at 37°C under 5% CO₂ conditions. A well that did not contain the sample or standard material but contained only the virus was used as a negative control, and a well that contained only cells was used as a positive control.

After the culture medium was removed, 100 μl of a neutral red solution was added to the plate to stain viable cells, followed by incubation for 2 hrs at 37°C under 5% CO₂ condition. After the supernatants were removed, 100 μl of a 1:1 mixture of 100% ethanol and 1% acetic acid was added to each well of the plate. After thorough mixing to dissolve all neutral red crystals eluted from stained cells, absorbance was measured at 540 nm. The negative control was

used as a blank, and ED₅₀ values (doses causing 50% cell growth inhibition) were calculated, where the cell growth of the positive control was set at 100%.

TABLE 8

	Conc. (ng/ml)	Specific activity (IU/mg)	Relative activity (%) for native IFN α
Native IFN α	100	4.24E+08	100
IFN α -40K PEG	100	2.04E+07	4.8
IFN α -PEG-albumin	100	2.21E+07	5.2
IFN α -PEG-Fc	100	1.19E+08	28.1
IFN α -PEG-DG Fc	100	1.09E+08	25.7
IFN α -PEG-recombinant AG Fc derivative	100	9.58E+07	22.6

5 As shown in Table 8, the IFN α -40K PEG decreased in activity to 4.8% of the native IFN α . Especially, as the size of the PEG moieties increased, a protein conjugate has improved serum stability but gradually decreased activity. Interferon alpha was reported to have in vitro activities of 25% when modified with 12-kDa PEG and about 7% when modified with 40-kDa PEG (P. Bailon et al., *Bioconjugate Chem.* 12: 195-202, 2001). That is, since a protein conjugate has a longer half-life but sharply decreases in biological activity as the molecular weight of PEG moieties increase, there is a need for the development of a protein conjugate having a longer serum half-life and a stronger activity. In addition, the IFN α -PEG-albumin conjugate displayed a weak activity of about 5.2% compared to the native IFN α . In contrast, the IFN α -PEG-Fc and IFN α -PEG-DG

Fc conjugates of the present invention exhibited a markedly improved relative activity of 28.1% and 25.7% compared to the native IFN α . Also, the conjugation of IFN α to the recombinant AG Fc derivative resulted in a similar increase 5 in activity. From these results, it is expected that interferon alpha conjugated to the immunoglobulin Fc fragment has a markedly increased serum half-life and greatly improved pharmaceutical efficacy in vivo.

10 <4-2> Comparison of the human growth hormone protein conjugates for intracellular activity

To compare the intracellular activity of the human growth hormone protein conjugates, the hGH-PEG-Fc, hGH-40K PEG and hGH-PEG-albumin were compared for intracellular activity.

15 Intracellular activities of the hGH conjugates were measured by an in vitro assay using a rat node lymphoma cell line, Nb2 (European Collection of Cell Cultures (ECACC) #97041101), which develops human growth hormone-dependent mitogenesis.

20 Nb2 cells were cultured in Fisher's medium supplemented with 10% FBS (fetal bovine serum), 0.075% NaCO₃, 0.05 mM 2-mercaptoethanol and 2 mM glutamin, and were further cultured in a similar medium not containing 10% FBS for 24 hrs. Then, the cultured cells were counted, and 25 about 2 \times 10⁴ cells were aliquotted onto each well of a 96-

well plate. The hGH-PEG-Fc, the hGH-40K PEG, the hGH-PEG-albumin, a standard available from the National Institute for Biological Standards and Controls (NIBSC) as a control, and native human growth hormone (HM-hGH) were diluted and added to each well at various concentrations, followed by incubation for 48 hrs at 37°C under 5% CO₂ condition. Thereafter, to measure cell proliferation activity by determining the cell number in each well, 25µl of the Cell Titer 96 Aqueous One Solution Reagent (Promega) was added to each well, and the cells were further cultured for 4 hrs. Absorbance was measured at 490 nm, and a titer for each sample was calculated. The results are given in Table 9, below.

TABLE 9

	Conc. (ng/ml)	Specific activity* (U/mg)	Relative activity (%) for native HM-hGH
Native hGH	100	2.71E+06	100
hGH (standard available from NIBSC)	100	2.58E+06	95.2
hGH-40K PEG	100	0.206E+06	7.6
hGH-PEG-albumin	100	0.141E+06	5.2
hGH-PEG-Fc	100	0.76E+06	28.1

Specific activity* = $1/ED_{50} \times 10^6$ (ED₅₀: protein amount required for 50% of maximum cell growth)

As shown in Table 9, also in the case of human growth hormone, the conjugation to 40-kDa PEG (hGH-40K PEG) resulted in a decrease in activity to about 7.6% of the native form, and the hGH-PEG-albumin conjugate displayed a

low in vitro activity that was about 5.2% of the native hGH. However, the hGH-PEG-Fc conjugate of the present invention markedly increased in relative activity to greater than 28% compared to the native hGH. From these 5 results, it is expected that human growth hormone linked to the immunoglobulin Fc fragment has a markedly increased serum half-life and a greatly improved in vivo pharmaceutical efficacy. In addition, it is believed that 10 the increased activity of the immunoglobulin Fc protein conjugates of the present invention is due to the increased serum stability and preserved binding affinity to receptors due to the immunoglobulin Fc or due to the space formed by the non-peptide polymer. These effects are predicted to be applicable to immunoglobulin Fc protein conjugates coupled 15 to other physiologically active proteins.

<4-3> Comparison of the G-CSF protein conjugates for intracellular activity

To compare the intracellular activity of the protein conjugates with a G-CSF derivative, the native G-CSF 20 (Filgrastim, Jeil Pharm. Co., Ltd.), ¹⁷Ser-G-CSF derivative, 20K PEG-G-CSF (Neulasta), 40K PEG-¹⁷S-G-CSF, ¹⁷Ser-G-CSF-PEG-albumin and ¹⁷S-G-CSF-PEG-Fc were compared for intracellular activity.

First, a human myeloid cell line, HL-60 (ATCC CCL-25 240, promyelocytic leukemia patient/36 yr old Caucasian

female), was cultured in RPMI 1640 medium supplemented with 10% FBS. The cultured cells were suspended at a density of about 2.2×10^5 cells/ml, and DMSO (dimethylsulfoxide, culture grade, Sigma) was added thereto at a final concentration of 5 1.25% (v/v). Then, 90 μ l of the cell suspension was seeded onto each well of a 96-well plate (Corning/low evaporation 96 well plate), thus providing a density of about 2×10^4 cells per well, and cultured in an incubator at 37°C with 5% CO₂ for about 72 hrs.

10 Each sample, whose protein concentration was determined using a G-CSF ELISA kit (R&D systems), was diluted with RPMI 1640 to an identical concentration of 10 μ g/ml, and further diluted two-fold with RPMI 1640 nineteen times. The serial two-fold dilutions were individually 15 added to each well containing HL-60 cells at a volume of 10 μ l, so that the concentration of each sample started at 1 μ g/ml. Then, the cells were cultured in an incubator at 37°C for 72 hrs.

20 The proliferation of HL-60 cells was assayed using Cell Titer 96TM (Cat. NO. G4100, Promega), and the increased cell number was determined by measuring absorbance at 670 nm.

TABLE 10

	ED ₅₀ (IU/mg)	Relative activity (%) for native G-CSF
Native G-CSF	0.30	100

¹⁷ Ser-G-CSF	0.26	115
G-CSF-20K PEG (Neulasta)	1.20	25
¹⁷ Ser-G-CSF-40K PEG	10.0	<10.0
¹⁷ Ser-G-CSF-PEG-albumin	1.30	23.0
¹⁷ Ser-G-CSF-PEG-Fc	0.58	51.7

As shown in Table 10, the immunoglobulin Fc protein conjugates coupled to a G-CSF derivative having an amino acid substitution, ¹⁷Ser-G-CSF, also displayed similar effects to native G-CSF-coupled protein conjugates. The 5 ¹⁷Ser-G-CSF-PEG was previously reported to have a relatively increased serum half-life but a decreased activity compared to nonpegylated ¹⁷Ser-G-CSF (Korean Pat. Laid-open Publication No. 2004-83268). Especially, as the size of the 10 PEG moieties increased, a protein conjugate had increased serum stability but gradually decreased activity. The ¹⁷Ser-G-CSF-40K PEG showed a very low activity of less than about 10% compared to the native form. That is, since a 15 protein conjugate has an extended serum half-life but a sharply decreased activity as the molecular weight of PEG moieties increases, there is the need for the development of a protein conjugate having a long serum half-life and strong activity. The ¹⁷Ser-G-CSF-PEG-albumin also showed a low activity of about 23% compared to the native G-CSF. In contrast, the ¹⁷Ser-G-CSF-PEG-Fc was greatly improved in 20 relative activity to more than 51% compared to the native G-CSF. From these results, it is expected that ¹⁷Ser-G-CSF linked to the immunoglobulin Fc fragment has a markedly increased serum half-life and a greatly improved

pharmaceutical in vivo efficacy.

<4-4> Cytotoxicity neutralization assay for the Fab' conjugates

An in vitro activity assay was carried out using the
5 Fab'-S-PEG-N-Fc and Fab'-N-PEG-N-Fc conjugates prepared in
Example 8 and 9 and the Fab'-S-40K PEG complex prepared in
Comparative Example 3. Through a cytotoxicity assay based
on measuring TNF α -mediated cytotoxicity, the Fab' conjugates
were evaluated to determine whether they neutralize TNF α -
10 induced apoptosis in a mouse fibroblast cell line, L929
(ATCC CRL-2148).

The Fab'-S-PEG-N-Fc and Fab'-N-PEG-N-Fc conjugate and
the Fab'-S-40K PEG complex were serially two-fold diluted,
and 100- μ l aliquots were placed onto wells of a 96-well
15 plate. rhTNF- α (R&D systems) and actinomycin D (Sigma) used
as an RNA synthesis inhibitor were added to each well at
final concentrations of 10 ng/ml and 1 μ g/ml, respectively,
incubated for 30 min in an incubator at 37°C with 5% CO₂,
and transferred to a microplate for assay. L929 cells were
20 added to each well at a density of 5 \times 10⁴ cells/50 μ l medium
and cultured for 24 hrs in an incubator at 37°C with 5% CO₂.
After the culture medium was removed, 50 μ l of MTT (Sigma)
dissolved in PBS at a concentration of 5 mg/ml was added to
25 each well, and the cells were further cultured for about 4
hrs in an incubator at 37°C with 5% CO₂. 150 μ l of DMSO was

added to each well, and the degree of cytotoxicity neutralization was determined by measuring the absorbance at 540 nm. As a control, the Fab' purified in the step 1 of Example 8 was used.

5 As shown in FIG. 13, all of the protein conjugates used in this test had a similar titer to the Fab'. These results indicate that, when a protein conjugate is prepared by linking an immunoglobulin Fc to a free cysteine residue near the N-terminus or C-terminus of a Fab' through PEG, the
10 Fab' exhibits a markedly increased serum half-life and a high in vivo activity.

<4-5> Complement-dependent cytotoxicity (CDC) assay

To determine whether the derivatives prepared in Examples and proteins corresponding to the constant regions 15 of immunoglobulins, expressed in the *E. coli* transformants and purified, bind to human C1q, an enzyme linked immunosorbent assay (ELISA) was carried out as follows. As test groups, immunoglobulin constant regions produced by the HM10932 and HM10927 transformants, deposited at the
20 Korean Culture Center of Microorganisms (KCCM) on Sep. 15, 2004 and assigned accession numbers KCCM-10597, KCCM-10588, and the derivatives prepared in the above Examples were used. As standards, a glycosylated immunoglobulin (IVIG-globulin S, Green Cross PBM) and several commercially
25 available antibodies used as therapeutic antibodies were

used. The test and standard samples were prepared in 10 mM carbonate buffer (pH 9.6) at a concentration of 1 μ g/ml. The samples were aliquotted into a 96-well plate (Nunc) in an amount of 200 ng per well, and the plate was coated 5 overnight at 4°C. Then, each well was washed with PBS-T (137 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.05% Tween 20) three times, blocked with 250 μ l of a blocking buffer (1% bovine serum albumin in PBS-T) at room 10 temperature for 1 hr, and washed again with the same PBS-T three times. The standard and test samples were diluted in PBS-T to a predetermined concentration and added to antibody-coated wells, and the plate was incubated at room 15 temperature for 1 hr and washed with PBS-T three times. Thereafter, 2 μ g/ml C1q (R&D Systems) was added to the plate and reacted at room temperature for 2 hrs, and the plate was washed with PBS-T six times. 200 μ l of a 1:1000 dilution of a human anti-human C1q antibody-peroxidase conjugate (Biogenesis, USA) in the blocking buffer was 20 added to each well and reacted at room temperature for 1 hr. After each well was washed with PBS-T three times, equal volumes of color reagents A and B (Color A: stabilized peroxide and Color B: stabilized chromogen; DY 25 999, R&D Systems) were mixed, and 200 μ l of the mixture was added to each well, followed by incubation for 30 min. Then, 50 μ l of a reaction termination solution, 2 M sulphuric acid, was added to each well. The plate was read

using a microplate reader (Molecular Device). Absorbance of standard and test samples was measured at 450 nm, and the results are given in FIGS. 14 and 15, respectively.

When immunoglobulin subclasses were compared with each other for complement activity in their immunoglobulin Fc fragment, the highest binding affinity to C1q was found in human immunoglobulin IgG1 (Fitzgerald), the next in IgG2 (Fitzgerald) and then IgG4 (Fitzgerald), indicating that there is a difference between subclasses in complement activity. The IVIG used in this test, which is a pool of IgG subclasses, exhibited a C1q binding affinity almost the same as the purified IgG1 because IgG1 amounts to most of the IVIG. Compared to these standards, with respect to changes in binding affinity to C1q by aglycosylation, IgG1 Fc having the strongest complement activity markedly decreased when aglycosylated. IgG4 Fc, known not to induce complement activation, rarely had binding affinity to C1q, indicating that the IgG4 Fc is used as an excellent recombinant carrier with no complement activity (FIG. 14).

To determine whether the carrier maintains its property of having no binding affinity to C1q even after being conjugated to a physiologically active peptide, IFN alpha-Fc conjugates were prepared using glycosylated Fc, enzymatically deglycosylated Fc and aglycosylated recombinant Fc as carriers for IFN alpha and were evaluated for their binding affinity to C1q. A glycosylated Fc-

coupled IFN alpha conjugate (IFN α -PEG-Fc: Glycosylated IgG1Fc) maintained a high binding affinity to Clq. In contrast, when interferon alpha was coupled to an Fc deglycosylated using PNGase F and other enzymes, the 5 resulting conjugate (IFN α -PEG-DGFc: Deglycosylated IgG1Fc) displayed a markedly decreased binding affinity to Clq, which was similar to that of the *E. coli*-derived aglycosylated Fc conjugate. In addition, when the IgG1 moiety of the aglycosylated IgG1 Fc-coupled interferon 10 alpha conjugate (IFN α -PEG-AGFcG1: Aglycosylated IgG1Fc) was exchanged with the IgG4 moiety, the resulting interferon conjugate (IFN α -PEG-FcG4 derivative 1: Aglycosylated IgG4Fc) was found to completely lose its binding affinity to Clq. When the IgG1 Fc moiety was exchanged with the IgG4 15 Fc monomer, the resulting conjugate (IFN α -PEG-FcG4 derivative 2: Aglycosylated IgG4Fc). These results indicate that such forms of the IgG4 Fc fragment are useful as excellent carriers not having the effector functions of antibody fragments (FIG. 15).

20 Industrial Applicability

As described hereinbefore, the pharmaceutical composition of the present invention greatly increases plasma half-lives of drugs. On the other hand, the protein conjugates overcome the most significant disadvantage of

conventional long-acting formulations, decreasing drug titers, thus having blood circulation time and in vivo activity superior to albumin, previously known to be most effective. In addition, the protein conjugates have no risk 5 of inducing immune responses. Due to these advantages, the protein conjugates are useful for developing long-acting formulations of protein drugs. The long-acting formulations of protein drugs according to the present invention are capable of reducing the patient's pain from frequent 10 injections, and of maintaining serum concentrations of active polypeptides for a prolonged period of time, thus stably providing pharmaceutical efficacy.

Further, the present method of preparing a protein conjugate overcomes disadvantages of fusion protein production by genetic manipulation, including difficult 15 establishment of expression systems, glycosylation different from a native form, immune response induction and limited orientation of protein fusion, low yields due to non-specific reactions, and problems of chemical coupling 20 such as toxicity of chemical compounds used as binders, thereby easily economically providing protein drugs with extended serum half-life and high activity.

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

To. Kyung-yoon Min

Hanmi Pharmaceutical Co., Ltd.
893-3, Hajeo-ri, Paltan-myeon, Hwaseong-si,
Gyeonggi-do, Korea

RECEIPT IN THE CASE OF AN ORIGINAL
issued pursuant to Rule 7.1 by the
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identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR : HM10932	Accession number given by the INTERNATIONAL DEPOSITORY AUTHORITY: KCCM-10597
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
<p>The microorganism identified under I above was accompanied by:</p> <p><input type="checkbox"/> a scientific description</p> <p><input type="checkbox"/> a proposed taxonomic designation</p> <p>(Mark with a cross where applicable)</p>	
III. RECEIPT AND ACCEPTANCE	
<p>This International Depository Authority accepts the microorganism identified under I above, which was received by it on Sep. 15. 2004. (date of the original deposit)¹</p>	
IV. INTERNATIONAL DEPOSITORY AUTHORITY	
<p>Name : Korean Culture Center of Microorganisms</p> <p>Address : 361-221, Yurim B/D Hongje-1-dong, Seodaemun-gu SEOUL 120-091 Republic of Korea</p>	<p>Signature(s) of person(s) having the power to represent the International Depository Authority or of authorized official(s):</p> <p>Date: Sep. 21. 2004</p> 

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depository authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depository authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depository authority.

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